

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
10 January 2002 (10.01.2002)

PCT

(10) International Publication Number
WO 02/02784 A1

(51) International Patent Classification⁷: C12N 15/74,
C12Q 1/68

MA 02446 (US). WOBBE, Richard, C.; 57 Spring Street,
Lexington, MA 02421 (US). BAILEY, David, A.; 68 Ho-
bart Street, Apartment 2, Brighton, MA 01235 (US).

(21) International Application Number: PCT/US01/16271

(22) International Filing Date: 18 May 2001 (18.05.2001)

(74) Agents: CHOKSI, Neepa, Y. et al.; Darby & Darby P.C.,
805 Third Avenue, New York, NY 10022-7513 (US).

(25) Filing Language: English

(81) Designated States (*national*): CA, JP.

(26) Publication Language: English

(30) Priority Data:
09/573,322 18 May 2000 (18.05.2000) US

(84) Designated States (*regional*): European patent (AT, BE,
CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC,
NL, PT, SE, TR).

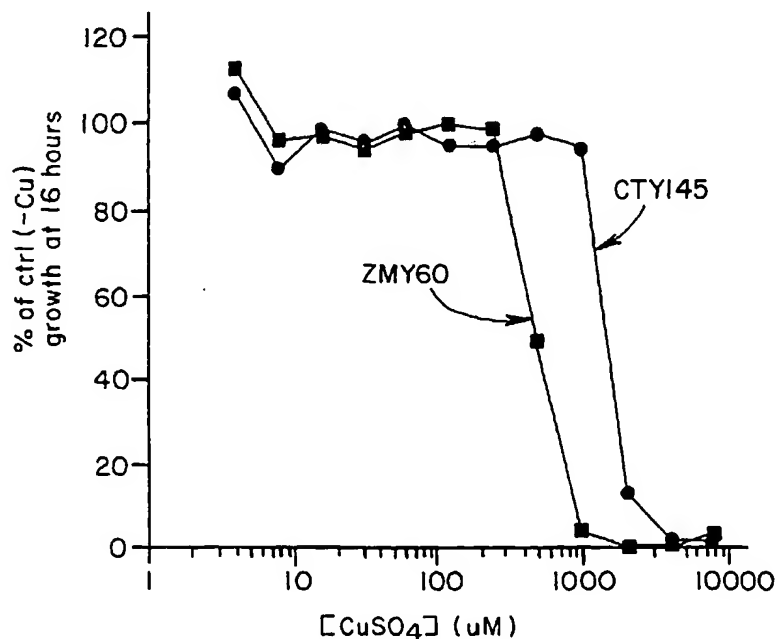
(71) Applicant: ANADYS PHARMACEUTICALS, INC.
[US/US]; 610 Lincoln Street, Waltham, MA 02451 (US).

Published:
— with international search report

(72) Inventors: BRADLEY, John, D.; 11426 Daykin Drive, St.
Louis, MO 63146 (US). THOMPSON, Craig, M.; 15 Col-
lege Avenue, Arlington, MA 02474 (US). MOORE, Jef-
frey, B.; 20 Chappel Street, Apartment A307, Brookline,

For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.

(54) Title: REGULATED GENE EXPRESSION IN YEAST AND METHODS OF USE



(57) Abstract: The invention provides novel yeast cells comprising genes whose expression can be modulated by growth in the presence or absence of metal ions, methods for making such yeast cells, and methods of using such yeast cells for determining the requirement for expression of particular genes for the growth or viability of the yeast cells. The invention also provides methods of using such yeast cells in the isolation, screening and analysis of candidate antifungal compounds.

REGULATED GENE EXPRESSION IN YEAST AND METHODS OF USE

This application is a continuation-in-part of U.S. Patent Application Serial
5 No. 09/404,066, filed September 23, 1999, which is a division of U.S. Patent Application
No. 09/138,024, filed August 21, 1998, now U.S. Patent No. 6,004,779, which claims
priority under 35 U.S.C. § 119 from U.S. Provisional Patent Application 60/056,719,
filed August 22, 1997, each of which are hereby incorporated by reference in its entirety.

10 Field of the Invention

The present invention relates to methods and compositions for regulated
expression of specific genes in *Saccharomyces cerevisiae*. The invention can be used to
identify and clone genes of interest and to identify antifungal agents using high-
throughput screening techniques. The invention also relates to the use of the
15 *Saccharomyces cerevisiae* strains of the invention in the isolation and analysis of
antifungal agents.

Background of Invention

The ability to regulate the expression of particular genes of interest is
20 important for many purposes, including, for example, (i) investigation of the biological
function of a particular gene product; (ii) design of variants of the gene product that are
tailored for different ends; and (iii) identification of agents that influence the activity of
the gene product, including, e.g., inhibitors or activators. The ease of performing genetic
and molecular manipulations in *S. cerevisiae* has made it an extremely useful
25 experimental organism for regulated expression of recombinant genes. However, many
gene expression systems based on *S. cerevisiae* are limited in their applicability by (i) the
degree of regulation that can be achieved, i.e., the extent to which genes can be turned

on and off, as well as the timing of these events; (ii) the relative stability of certain gene products, which makes it difficult to quickly deplete the cell of a gene product; and (iii) potential metabolic side effects of the procedures used to trigger or initiate changes in gene expression.

5 Thus, there is a need in the art for *S. cerevisiae* expression systems in which gene expression can be tightly and efficiently regulated, with respect to both transcription of the gene and accumulation of the protein product.

Summary of the Invention

10 The present invention encompasses yeast strains in which expression of a particular protein (the "subject" protein) can be tightly regulated. The invention provides *Saccharomyces cerevisiae* cells in which expression of the subject protein can be repressed by exogenous metal. These cells comprise, for example:

(i) a first gene encoding a transcriptional repressor protein, the
15 expression of which has been placed under the control of a metal ion-responsive element, wherein expression of the repressor protein is stimulated by the addition of a metal ion to the growth medium of the cells;

(ii) a second gene encoding a subject protein, wherein
expression of the subject protein is controlled by a promoter, the activity of which is
20 inhibited by said repressor protein; and

(iii) a third gene encoding a biomineralization protein, wherein
the third gene is inactivated and wherein inactivation of the third gene enhances the
transcriptional response of the metal-responsive element to added metal ions.

In a preferred embodiment, the first gene is ROX1; the second gene is
25 controlled by an ANB1 promoter; and the third gene is SLF1.

In another embodiment, the yeast cell comprises a fourth gene encoding

a protein that targets ubiquitin-containing polypeptides for degradation, where the fourth gene is placed under the control of a metal ion-responsive element. In a preferred embodiment, the fourth gene is the UBR1 gene.

The invention further comprises yeast cells in which expression of the subject protein is stimulated by exogenous metal ions. These cells comprise:

- (i) a first gene encoding a subject protein, wherein expression of the gene encoding the subject protein is under the control of a metal ion-responsive element and is stimulated by the addition of a metal ion to the growth medium of the cells; and
- (ii) a second gene encoding a biomineralization protein, wherein the second gene is inactivated and wherein inactivation of the second gene enhances the transcriptional response of the metal-responsive element to added metal ions.

In a preferred embodiment, the metal-responsive element is the Sc3451 promoter and the second gene is SLF1.

In another aspect, the invention relates to a method for the introduction of a subject gene under the control a predetermined promoter DNA sequence into a yeast cell genome, comprising the steps of providing a shuffled gene fragment, where the fragment comprises a restriction enzyme cleavage sequence, ligating the shuffled gene fragment into a vector, where the ligation results in the shuffled gene fragment being operably linked to a predetermined transcriptional control DNA sequence, cutting the vector with a restriction enzyme specific for the restriction enzyme cleavage sequence to yield a linearized vector, and transforming a yeast cell with the linearized vector.

The invention also provides methods for repressing or activating expression of a gene encoding a subject protein in *S. cerevisiae* to a predetermined level, comprising culturing the strains described above in the presence of metal, wherein the

metal is present at sufficient concentration to activate the metal-responsive element so as to achieve the predetermined level of repression or activation of the gene.

In a further embodiment, the invention also encompasses methods for generating the yeast strains of the invention comprising:

- 5 (a) generating a yeast cell comprising
- (i) a first gene encoding a transcriptional repressor protein whose expression is under the control of a metal ion-responsive element, wherein expression of said first gene encoding said repressor protein is stimulated by the addition of a metal ion to growth medium of said yeast cell;
 - 10 (ii) a second gene encoding a subject protein, wherein expression of said second gene encoding said subject protein is controlled by a transcriptional control sequence whose activity is inhibited by said repressor protein; and
 - (iii) a third gene encoding a biomineralization protein, wherein said third gene is inactivated and wherein inactivation of said third gene enhances
15 transcriptional response of said metal ion-responsive element to metal ions in said growth medium of said yeast cell;
- (b) culturing the yeast cell in a growth medium comprising metal ions, wherein said metal ions are present in sufficient concentration to activate said metal ion-responsive element to a level which will result in said predetermined level of repression
20 of expression of said subject gene;
- (c) assessing whether the rapid depletion of the second gene from the yeast cell leads to inhibition of cell growth or cell death.

In a preferred embodiment the identifies the target gene as an essential target gene.

In another embodiment the invention is directed to methods of screening a candidate antifungal compound for interaction with an essential target gene comprising:

(a) generating a regulated yeast strain comprising a regulated essential target gene of the invention;

5 (b) establishing a concentration of metal ion at which the growth or viability of the regulated yeast strain ceases;

(c) generating a serial dilution of metal ion in yeast growth media;

(d) culturing the regulated yeast strain in the serially diluted growth media, wherein the serial dilution leads to a dose-dependent modulation of expression of the regulated essential target yeast gene product;

10

(e) screening the serially diluted cultures for altered sensitivity of the strain to the candidate antifungal compound;

(f) determining the metal ion concentration present in a culture demonstrating altered sensitivity to the candidate antifungal compound;

15 (g) comparing the metal ion concentration of step (b) with the metal ion concentration of the culture determined in step (f), and identifying a candidate antifungal compound for which a lower concentration of metal ion is required to eliminate growth or viability in step (f) as compared to step (b).

The method of screening may screen a single candidate or a plurality of candidate antifungal compounds. When a plurality of candidates screened, the screen is selected from the group consisting of screening together in a single assay and screening individually using multiple simultaneous individual detecting steps.

20

The invention also encompasses methods of rapidly cloning a DNA complementary to an essential target gene comprising:

(a) generating a regulated yeast strain comprising a regulated essential target gene of the invention;

(b) establishing a concentration of metal ion at which the growth or viability of the regulated yeast strain ceases;

5 (c) transforming the regulated yeast strain with a DNA to be tested for complementation;

(d) culturing the transformed regulated yeast strain in growth media containing a concentration of metal ion as established in step (b);

(e) determining the ability of the DNA to complement the regulated
10 essential target gene, wherein growth or viability of the regulated yeast strain establishes complementation; and

(f) cloning the complementary DNA.

In a further embodiment, the DNA is selected from the group consisting of genes from another organism, mutant DNA and DNA fragments which can be
15 generated from either genomic or cDNA libraries. In a further embodiment, the DNA is selected from an organism selected from the group consisting of human, mouse, mammal, drosophila and mycete.

The invention further encompasses methods of determining the antifungal effect of an antifungal compound comprising:

20 (a) generating a regulated yeast strain comprising a regulated essential target gene of the invention;

(b) establishing a concentration of metal ion at which the growth or viability of the regulated yeast strain ceases;

(c) culturing the regulated yeast strain in growth media containing the

concentration of metal ion as established in step (b);

(d) determining the phenotype associated with the culture of step (c) that is depleted of the essential target gene;

(e) culturing a yeast strain in growth media with a candidate antifungal compound;

(f) determining the phenotype associated with the culture of step (e) that is treated with the candidate antifungal compound;

(g) comparing the phenotypes determined in steps (d) and (f) to determine the antifungal effect of the antifungal compound.

In a further embodiment, the phenotypes are determined by

(i) incubating the cultures with radio-labeled macromolecular building-blocks;

(ii) establishing a level of incorporation of the radio-labeled macromolecular building blocks for each culture; and

(iii) analyzing the macromolecular products generated in each culture.

Brief Description of the Drawings

Figure 1 is a graphic illustration of the growth of yeast strains CTY145 and ZMY60 in increasing concentrations of copper sulfate. CTY145 is four-fold more tolerant to copper than ZMY60.

Figures 2A-C are schematic illustrations of the single- or double-round PCR strategy of the present invention that is used to construct a copper-inducible promoter element for any gene of interest. For single round PCR, primer pairs 1 and 2a or 2b are used to produce the transforming DNA. For the double-round PCR, primer

pairs 2a or 2b are used with additional primers corresponding to sequences located 400-1000 bp upstream or downstream of the ATG start site to prepare long primers which are then used in a second round of PCR to produce transforming DNA.

Figure 3 is a schematic illustration of a PCR primer design strategy that can be used to render a gene encoding a protein of interest repressible by LexA. The upper panel shows the structure of the promoter complex that can be inserted upstream of the gene of interest. The middle panel shows the location of the sequences corresponding to the PCR primers that can be utilized. The lower panel shows the resulting promoter cassette fused to the gene of interest which can be introduced into yeast.

Figures 4A-D illustrate the nucleotide sequence of the ZM195 plasmid (SEQ ID NO:20).

Figures 5A-D illustrate the nucleotide sequence of the ZM197 plasmid (SEQ ID NO:21).

Figure 6 is a schematic illustration of the PCR strategy used to generate shuffled genes for transformation into *S. cerevisiae*.

Figure 7 is a schematic illustration of the transformation mechanism by which shuffled genes are introduced into *S. cerevisiae*.

Figure 8 is a restriction map of the pCU19Srf vector (SEQ ID NO:22), showing the unique restriction enzyme cutting sites.

Figure 9 is a photograph of cell filtrates demonstrating the effect of deleting the SLF1 gene on biomineralization by yeast.

Figure 10 is a graph demonstrating the effect of copper sulfate on the growth of yeast strains which express and do not express the SLF1 gene.

Figure 11 is a graph showing growth curves for control and recombinant yeast strains which were grown in the presence of copper sulfate.

Figure 12 is an schematic example of a drug sensitivity dilution assay.

Figure 13 is an example of the set up for a drug sensitivity plate assay.

5 Figure 14 is a photograph showing the effect of copper sulfate on the growth of yeast strains that express wild type, mutated and Cu-regulated forms of the BOS1 gene.

Figure 15 provides Northern blot analysis showing the effect of copper sulfate on tRNA synthesis in yeast strains with the RPC34 and TAF145 Cu-regulated.

10 Figure 16 is a series of photographs of microtiter plates demonstrating the effect of increasing concentrations of copper sulfate on yeast strain sensitivity to tunicamycin in strains which express Cu-regulated ALG-7 (panel a) and CHS3 (panel b) and in wild type (panel c).

15 Figure 17 is a series of photographs demonstrating the effect of increasing concentrations of copper sulfate on yeast strain sensitivity to various antifungal compounds.

Detailed Description of the Invention

20 All patents, patent applications, publications and other materials cited herein are hereby incorporated by reference in their entirety. In the case of inconsistencies, the present description, including definitions, is intended to control.

As used herein, the term "transcriptional repressor protein" refers to a protein which either binds directly to a transcriptional control sequence or which binds

in association with other proteins or cofactors to a transcriptional control sequence, resulting in the repression of transcription of the protein encoding nucleotide sequence or sequences to which the transcriptional control sequence is operably linked.

As used herein, the term "transcriptional control sequence" refers to DNA sequences, such as initiator sequences, enhancer sequences, and promoter sequences, which induce, repress, or otherwise control the transcription of protein encoding nucleic acid sequences to which they are operably linked.

As used herein, the term "metal-ion responsive element" refers to a transcriptional control sequence which is activated when in the presence of an appropriate concentration of metal ions.

As used herein, the term "inactivated", when referring to a gene, means that the gene cannot be transcribed, either due to deletion of the gene from a genome or by disruption of its coding or regulatory sequences.

As used herein, the term "biomineralization protein" refers to a protein that promotes or catalyzes the conversion of ionic copper to a form insoluble in water, such as CuS.

As used herein, the term "shuffled gene fragment" refers to the nucleotide sequence around the ATG initiation codon of a gene, from about 400 nucleotides upstream of (i.e., 5' to) the ATG initiation codon of the gene to about 400 protein coding nucleotides downstream of (i.e., 3' to) the ATG initiation codon of the gene, wherein the orientation of the upstream and downstream sequences have been changed such that the ATG initiation codon and the approximately 400 downstream protein coding nucleotides that follow the ATG codon in the wild-type gene are upstream to the approximately 400 noncoding nucleotides normally found adjacent and upstream of the ATG initiation

codon. The shuffled gene fragment will typically contain a restriction enzyme cleavage sequence between the rearranged coding and noncoding nucleotide sequences.

As used herein, the term "restriction enzyme cleavage sequence" refers to a specific nucleotide sequence which is specifically recognized and cleaved by one or
5 more restriction endonuclease enzymes.

As used herein, the term "operably linked" refers to the covalent attachment, typically of a transcriptional control sequence to a protein encoding nucleotide sequence, such that transcription of the protein encoding nucleotide sequence is regulated or controlled by the transcriptional control sequence.

10 As used herein, the term "linearized vector" refers to the cleavage product of circular double stranded DNA molecule, or vector, which has been cleaved at a single site, yielding a linear double stranded DNA molecule.

As used herein, "inhibition" refers to a reduction in the parameter being measured, whether it be fungal growth, DNA transcription, protein synthesis, etc.. The
15 amount of such reduction is measured relative to a standard (control). Because of the multiple interactions of many *Saccharomyces* proteins in cell division, growth and cell cycle regulation, the particular target gene product for detection may vary with respect to the particular screening assay employed.

As used herein, "reduction" is defined as a decrease of at least 25%
20 relative to a control, preferably of at least 50%, and most preferably of at least 75%.

As used herein, "growth" refers to the normal growth pattern of *S. cerevisiae*, i.e., to a cell doubling time of 60-90 minutes during the log phase of growth. In rich media, wild-type *S. cerevisiae* strains have a doubling time of 90 minutes. Growth of the cells may be measured by following the optical density of cells in liquid media.

An increasing optical density indicates growth. Growth can also be measured by colony formation from single cells on solid media plates.

As used herein, "viability" refers to the ability of the *S. cerevisiae* cells to resume growth following a treatment of the cells which results in cessation of growth. Examples of such treatments resulting in cessation of growth include, but are not limited to, transient inactivation of a gene product required for growth or treatment with an antifungal drug. One typical means by which viability is measured is by testing the ability of cells to form colonies on solid media plates following removal of the treatment which resulted in a cessation of growth. Cells that fail to form colonies are considered inviable.

As used herein, "cidal" is defined as a rapid loss in viability. Rapid is defined as a population of cells losing viability with a measured half-life of at least about 2 hours or less.

As used herein, "candidate inhibitor" is any compound with a potential to inhibit *Saccharomyces cerevisiae* growth or viability and may be used interchangeably with the term "candidate antifungal".

The present invention encompasses methods and compositions for regulating the expression of a gene of interest in *Saccharomyces cerevisiae*. The invention provides recombinant yeast strains which comprise:

- (i) a gene encoding a transcriptional repressor protein, the expression of which gene has been placed under the control of a metal ion-responsive element, so that expression of the gene encoding the repressor protein is stimulated by the addition of a metal ion to the growth medium of the cells;
- (ii) a gene encoding a protein of interest, the expression of which gene is inhibited by the repressor protein described in (i); and
- (iii) one or more genes involved in metal ion metabolism that have either been inactivated or overexpressed, depending on the gene, to enhance the

transcriptional response to added metal ion.

In the above yeast cells (a large number of such clonal cells being collectively designated "repressing strains"), the gene of interest is expressed in the absence of added metal ion. When it is desired to decrease or eliminate expression of the gene of interest, metal ions are added to the medium, which stimulates expression of the repressor to a degree that is dependent upon the concentration of added metal ions and represses transcription of the gene of interest.

The invention also encompasses yeast cells (a large number of such clonal cells being collectively designated "inducing strains") in which: (i) the gene of interest is operably linked to a metal ion-inducible transcriptional control sequence, so that expression of the gene of interest is directly stimulated by addition of metal ions to the medium; and (ii) one or more genes involved in metal ion metabolism that have either been inactivated or overexpressed, depending on the gene, to enhance the transcriptional response to added metal ion.

The choice of yeast strain in which the above manipulations are performed is important in practicing the invention. Suitable strains are those that tolerate the addition of metal ions to their culture medium at a sufficient concentration, and for a sufficient time period, to allow maximal expression of metal-inducible genes while maintaining cell viability and metabolism. Preferably, the growth rate of the strain should remain substantially unaffected for at least about 16 h after the addition of at least 750 mM copper sulfate, most preferably at least 1 mM copper sulfate. In addition, the strain should grow well and should be auxotrophic for common nutrients such as histidine, leucine, and uracil, to enable the use of, e.g., HIS3, LEU2, and URA3 as markers for genetic insertions. Suitable yeast strains include without limitation CTY145

(ATCC #74466) and S288C (ATCC #26108).

In some embodiments, the repressing strains of the invention further comprise a gene encoding a protein that targets ubiquitin-containing polypeptides for degradation via the ubiquitin degradation pathway, which, similar to the repressor gene, is expressed under the control of a metal ion-responsive regulatory element. In these embodiments, the gene of interest is expressed as a fusion protein, which contains at its amino terminus additional amino acids comprising a sequence that targets the polypeptide for the ubiquitin degradation pathway. In this manner, addition of metal ions to the medium also stimulates degradation of the protein of interest by the ubiquitin pathway, thereby depleting the protein from the cell. It will be understood, however, that some proteins of interest cannot be expressed in functional form as ubiquitin-targetable fusion proteins. Furthermore, overexpression of a ubiquitin-pathway gene may exert pleiotropic and potentially deleterious effects. Accordingly, the invention also encompasses repressing strains that do not overexpress a ubiquitin pathway protein and in which the gene of interest is not expressed as a fusion protein.

In practicing the invention, any metal ion-responsive transcriptional control element may be used, including without limitation DNA sequences comprising the binding site for the ACE1 protein, which has been identified as the sequence spanning nucleotides -105 to -148 of the CUP1 (metallothionein) promoter (Huitbregtse et al., *Proc.Natl.Acad.Sci.USA* 86:65, 1989). Metal ion-responsive elements may be used singly or in tandem repeats, in direct or reverse orientation relative to a transcription start site, and may be combined with any compatible promoter such as, e.g., the HIS3 promoter. In conjunction with these elements, any suitable metal ion may be used to stimulate expression, including without limitation Ag, Cu, Cd, Ni, Zn, and Fe ions.

Suitable repressor proteins for use in the invention include without limitation ROX1, a heme-induced repressor of hypoxic genes (Genbank accession number #X60458) (Deckert et al., *Genetics* 139:1149, 1995), LexA-CYC8 fusion proteins and LexA-TUP1 fusion proteins (Redd et al., *Cell* 78:709, 1992). It will be understood that the choice of promoter sequences to be placed upstream of the gene of interest will be determined by the particular repressor used. For example, when ROX1 is the repressor, the promoters directing expression of the gene of interest may be derived from, e.g., the ANB1, HEM13, ERG11, or OLE1 genes. The sequences of these genes are disclosed under the following Genbank accession numbers: #M23440 (ANB1); #S81592 (HEM13); #U10555, U00093 (ERG11); and #U42698, #J05676 (OLE1). When the repressor contains bacterial LexA domains, the promoters directing the expression of the gene of interest may comprise sequences derived from the LexA operator. The sequence of the LexA operator is 5'-TACTGATGTACATACAGTA-3' (Tzamarias et al., *Nature* 369:758, 1994) (SEQ ID NO:1); a synthetic LexA operator may also be employed, comprising the sequence:

5'-TCGAGTACTGTATGTACATACAGTACCATGACATACATGTATGTCATGAGCT-3'
(U.S. Patent No. 4,833,080) (SEQ ID NO:2).

The genes involved in metal ion metabolism that may be inactivated to form the yeast strains of the present invention include without limitation SLF1, which is involved in the biomineralization pathway of copper (Genbank accession number U30375) (Yu et al., *Mol.Cell.Biol.* 16:2464, 1996). In the case of SLF1, inactivation of the gene slows the depletion of copper from the growth medium and thereby enhances the transcriptional response of the repressor-encoding gene to the added copper ions. The result is an increase in the time period in which a consistent copper regulation of

gene expression can be maintained. Alternatively, genes encoding proteins such as, e.g., CTR1 (a metal ion transporter) can be overexpressed to increase the sensitivity of the transcriptional apparatus to the added metal ion (Dancis et al., *J. Biol. Chem.* **269**:25660, 1994).

5 In the embodiments in which a ubiquitin-pathway protein is expressed under metal ion control, any ubiquitin-pathway protein may be expressed that will stimulate the degradation of an appropriately amino terminal tagged protein of interest. In one embodiment, the ubiquitin pathway protein that is linked to a metal ion-responsive element is UBR1 and the amino terminal tag is a hybrid sequence comprising, in amino-
10 terminal-to-carboxyl terminal direction, ubiquitin and a 31-amino acid segment of the lac repressor protein (LacI), and may additionally include one or more epitope tags (Park et al., *Proc.Natl.Acad.Sci. USA* **89**:1249, 1992). In this embodiment, the hybrid protein (containing at its carboxyl terminus the protein of interest) is rapidly de-ubiquinated by yeast enzymes, and the resulting hybrid protein (containing an arginine residue at its
15 amino terminus) is re-ubiquinated by the UBR1 protein (in the presence of a metal ion) and targeted for degradation.

Moqtaderi et. al., *Nature* **383**:188, 1996, disclose a haploid yeast strain (ZMY60) carrying integrated copies of the ROX1 and UBR1 genes which were placed under the control of the ACE1 promoter. Into this genetic background, a plasmid
20 containing the ANB1 promoter driving expression of an in-frame fusion of ubiquitin, arginine, lacI, hemagglutinin epitopes and the full length gene of interest was introduced. Addition of 500 mM cupric sulfate (CuSO₄) to the medium resulted in the repression of transcription of the gene of interest by ROX1 and rapid degradation of the ubiquitin-tagged protein. However, this strain is relatively genetically unstable resulting in

frequent reversion to a copper-insensitive phenotype, is highly sensitive to the toxic effects of copper ions at concentrations above 250 mM, and responds to copper ions for a relatively short time (in part, due to depletion of copper ions from the medium by biomineralization). The yeast strains of the present invention, by contrast, tolerate
5 concentrations of copper ions of 1 mM or greater for extended periods of time. Furthermore, the yeast strains of the present invention exhibit more stable phenotypes, due to the use of methods which employ double-crossover events for integration of engineered genes into the yeast genome (see, e.g., Examples 3 and 4 below).

In one set of embodiments, the invention provides a CTY145-based yeast
10 strain in which: (i) the native ROX1 gene promoter has been replaced with a promoter comprising a hybrid HIS3 promoter-ACE1 binding site; (ii) the native SLF1 gene has been deleted; and (iii) the gene of interest is controlled by an ANB1 promoter. Features (i)-(iii) are preferably achieved using a double-crossover strategy. In an alternate embodiment, the CTY145 strain has been modified as in (i) and (ii) above, and, in
15 addition, (iii) the native UBR1 gene promoter has been replaced with a promoter comprising a hybrid HIS3 promoter-ACE1 binding site; and (iv) a sequence which comprises an ANB1 promoter followed by a sequence encoding a hybrid polypeptide comprising ubiquitin, a LacI fragment, and an epitope tag is fused to the 5' end of the protein-coding sequence of a gene of interest.

20 In another set of embodiments, the invention provides a CTY145-based yeast strain in which: (i) a gene has been introduced comprising a hybrid HIS3 promoter-ACE1 binding site placed upstream of sequences encoding a CYC8-LexA fusion protein; (ii) the native SLF1 gene has been deleted; and (iii) a gene of interest is controlled by a promoter comprising a LexA operator. Features (i)-(iii) are preferably achieved using a

double-crossover strategy. In an alternate embodiment, the CTY145 strain has been modified as in (i) and (ii) above, and, in addition, (iii) the native UBR1 gene promoter has been replaced with a promoter comprising a hybrid HIS3 promoter-ACE1 binding site; and (iv) a sequence which comprises a LexA operator-containing promoter followed
5 by a sequence encoding a hybrid polypeptide comprising ubiquitin, a LacI fragment, and an epitope tag is fused to the 5' end of the protein-coding sequence of a gene of interest.

In another set of embodiments, the invention provides a CTY145-based yeast strain in which (i) the gene of interest is controlled by the Sc3451 promoter and (ii) the native SLF1 gene has been deleted. The Sc3451 promoter was constructed by cloning
10 a n A C E 1 b i n d i n g s i t e (5 ' - TAAGTCTTTTTTGCTGGAACGGTTGAGCGGAAAAGACGCATC-3') (SEQ ID NO:3) upstream of the TATAA sequence at an EcoRI site in plasmid YIp55-Sc3370 (Struhl et al., *Mol. Cell Biol.* 7:104, 1987).

15 METHODS

In practicing the present invention, many conventional techniques in molecular biology, microbiology, and recombinant DNA, are used. Such techniques are well known and are explained fully in, for example, *Current Protocols in Molecular Biology*, Volumes I, II, and III, 1997 (F.M. Ausubel ed.); Sambrook et al., 1989,
20 *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; *DNA Cloning: A Practical Approach*, Volumes I and II, 1985 (D.N. Glover ed.); *Oligonucleotide Synthesis*, 1984, (M.L. Gait ed.); *Nucleic Acid Hybridization*, 1985, (Hames and Higgins); *Transcription and Translation*, 1984 (Hames and Higgins eds.); *Animal Cell Culture*, 1986 (R.I. Freshney

ed.); *Immobilized Cells and Enzymes*, 1986 (IRL Press); Perbal, 1984, *A Practical Guide to Molecular Cloning*; the series, *Methods in Enzymology* (Academic Press, Inc.); and *Methods in Enzymology* Vol. 154 and Vol. 155 (Wu and Grossman, and Wu, eds., respectively).

5 Insertion of nucleic acids (typically DNAs) comprising the sequences of the present invention into a vector is easily accomplished when the termini of both the DNAs and the vector comprise compatible restriction sites. If this cannot be done, it may be necessary to modify the termini of the DNAs and/or vector by digesting back single-stranded DNA overhangs generated by restriction endonuclease cleavage to produce blunt
10 ends, or to achieve the same result by filling in the single-stranded termini with an appropriate DNA polymerase.

 Alternatively, any site desired may be produced, e.g., by ligating nucleotide sequences (linkers) onto the termini. Such linkers may comprise specific oligonucleotide sequences that define desired restriction sites. Restriction sites can also
15 be generated by the use of the polymerase chain reaction (PCR). See, e.g., Saiki *et al.*, 1988, *Science* 239:48. The cleaved vector and the DNA fragments may also be modified if required by homopolymeric tailing.

 The nucleic acids may be isolated directly from cells. Alternatively, the polymerase chain reaction (PCR) method can be used to produce the nucleic acids of the
20 invention, using either chemically synthesized strands or genomic material as templates. Primers used for PCR can be synthesized using the sequence information provided herein and can further be designed to introduce appropriate new restriction sites, if desirable, to facilitate incorporation into a given vector for recombinant expression.

 Methods for yeast transformation, integration of genes into the yeast

genome, and growth and selection of yeast strains are fully described in, e.g., *Current Protocols in Molecular Biology*, Vols. 1 and 2, Ausubel et al., eds., John Wiley & Sons, New York (1997). The use of URA3 for the production of multiply disrupted yeast strains is disclosed in Alani et al., *Genetics* **116**:541, 1987.

5 A preferred method for the transformation of *S. cerevisiae* is as follows. Yeast strains are cultured overnight in YPD (yeast extract, peptone, dextrose) medium at about 30°C. The resulting culture is diluted to an A₆₀₀ of about 0.2 in about 200 ml YPD medium and incubated at about 30°C until the A₆₀₀ reaches approximately 0.8. The cells are pelleted by centrifugation and are washed in about 20 ml sterile water. The
10 pelleted yeast cells are then resuspended in about 10 ml TEL (10mMTris pH7.5, 1 mM EDTA, 0.1 M LiAcetate pH 7.5) buffer. The cells are pelleted by centrifugation and again resuspended in about 2 ml TEL. About 100 mg of well-sheared single stranded DNA and plasmid DNA are added to an eppendorf tube. To this tube is added about 100 ml of competent yeast cells, followed by mixing. To the cell/DNA mixture is added
15 about 0.8 ml of 40% PEG-3350 in TEL, followed by thorough mixing. This mixture is incubated for about 30 minutes at 30°C, followed by a heat shock for 20 minutes at 42°C. The mixture is centrifuged to remove the supernatant and pellet the cells. The yeast cell pellet is washed with about 1 ml TE, pelleted again by centrifugation, and then plated on selective media.

20 A preferred method for the extraction of genomic DNA from *S. cerevisiae* for PCR is as follows. A 5 ml overnight yeast strain culture grown in YPD at 30°C is spun out by centrifugation and washed once in 1 ml Tris pH 7.5/1 mM EDTA (TE) buffer. The cells are pelleted again by centrifugation and resuspended in 0.2 ml Extraction Buffer (2% Triton X100, 1% SDS, 100 mM NaCl, 10mM Tris pH 7.5 and 1mM EDTA) plus

0.2 ml phenol/chloroform/isoamyl alcohol. About 0.3 g of acid washed glass beads are added. This mixture is vortexed (i.e., agitated vigorously) for 30 minutes. 0.2 ml TE buffer is then added. The mixture is centrifuged and the aqueous phase is removed. The DNA is precipitated from the aqueous phase with two volumes of ethanol. The precipitate
5 is pelleted by microcentrifugation, and resuspended in 50 µl TE plus 5 mg/ml RnaseA enzyme. The resulting preparation can be diluted to a desired concentration, or used directly for PCR reactions.

For assaying the effects of copper ions on recombinant yeast strains, wild type and recombinant strains are grown in 5 ml of CSM media on a roller drum
10 incubation apparatus at 30°C for 18 to 20 hours. Cultures are diluted to an A_{600} (absorbance of light at 600 nm) of about 0.02 in 5 ml CSM media without or with various concentrations of CuSO_4 (10 mM, 50 mM, 100 mM, 250 mM, 1mM and 2 mM) for 18 to 20 hours. The A_{600} of the various samples is read and recorded.

Yeast strains are tested by a time course in the presence and absence of
15 copper to determine if the depletion of target gene product is fungistatic (i.e., inhibitory to growth) or fungicidal (i.e., yeast killing). Cultures are started from a single yeast colony in CSM media (5 ml) and grown at 30°C for 18 -20 hours in a roller drum. Cultures are diluted in fresh media to a final volume of 10 ml at an A_{600} of about 0.25 and allowed to grow at 30°C for 1 hour. The cultures are split into two aliquots and 1 mM CuSO_4 is
20 added to one of the aliquots. A sample of 300 µl is immediately taken from each culture aliquot as the zero time point. Other similar samples are taken at 1, 3, 5, 7 and 24 hours after CuSO_4 addition. Alternatively, the cultures can be diluted to an A_{600} of about 0.1 and allowed to grow for 3 hours, at which time the cultures are diluted again to an A_{600} of about 0.02, after which 1 mM CuSO_4 is added. To measure the absorbance of a yeast

culture, typically two-hundred microliters of each sample is taken and added to a 96-well flat bottom polystyrene plate, which is then inserted into a plate reader where the absorbance at 595 nm is measured. A growth curve can be generated from these readings.

5 When plating cells on YPD medium for analysis of CFU number, typically 100 ml of each sample is serially diluted in 900 ml sterile water. Plating dilutions for yeast cultured without copper ions and for wild type yeast cultured in the presence of copper ions are from 10^{-3} to 10^{-6} . Plating dilutions for time points 0, 1, 3, 5, and 7 hrs for recombinant yeast cultured in the presence of copper ions range from 10^{-2} to 10^{-5} . Plating
10 dilutions for any 24 hour time points for yeast cultured in the presence of copper ions ranges from undiluted to 10^{-2} .

Typically, about one-hundred microliters of each dilution is plated on YPD agar plates and incubated at 30°C for 48 hrs. Colonies are counted and recorded. Calculations are made to convert colony counts to CFU/ml of original culture medium.

15

APPLICATIONS

The yeast strains of the present invention find use for:

(i) *Rapid and efficient determination of whether a particular gene of interest can serve as a potential target for discovery of antifungal drugs.* The present
20 invention encompasses methods of identifying target genes useful in the discovery of antifungal agents. Identification of potential targets is carried out by assessing whether the rapid depletion from yeast cells of a particular gene product (using the "repressing strains" described above) leads to inhibition or slowing of cell growth, or cell death. Since the most effective and preferred antifungal drugs are those whose effect is rapidly
25 fungicidal, a gene product whose depletion leads to cell death is a preferred potential

target for a candidate inhibitor of fungal growth, *i.e.* an antifungal drug. Because the degree of the reduction in the amount of the gene product can be controlled by the concentration of metal added, it is further possible to determine the degree of reduction of the gene product necessary to cause cell death.

- 5 Various methods can be used to determine whether the product of a gene is essential to the survival of *S. cerevisiae* and thus essential to the establishment or maintenance of an infection. The identification of the essential character of a gene provides additional information regarding its function and allows selection of genes for which the product constitutes a target of interest for an antifungal agent or substance.
- 10 Examples of these methods are summarized briefly below. These methods are described in the following works, each of which are hereby incorporated by reference herein:
- Guthrie C. and Fink G.R. eds. Methods in Enzymology, Vol. 194, 1991, 'Guide to Yeast Genetics and Molecular Biology', Academic Press Inc.
 - Rose A.H., A.E. Wheals and J.S. Harrison eds. The Yeasts, Vol. 6, 1995, 'Yeast
 - 15 Genetics', Academic Press Inc.
 - Ausubel F. et al. eds. Short Protocols in Molecular Biology, 1995, Wiley.
 - Brown A.J.P. and Tuite M.F. (eds) Methods in Microbiology, Vol. 26, 1998, 'Yeast Gene Analysis' Academic Press Inc.

 Depending on the circumstances, one of the methods described may be

20 used, depending on the desired result. In particular, it is possible to proceed by a method of either direct inactivation of the gene or transitory inactivation of the gene. In the yeast *S. cerevisiae*, the method used most generally comprises inactivation of the gene of interest at its site within the chromosome of the yeast. The wild type allele is inactivated by insertion of a genetic marker (for example a gene for auxotrophy or a resistance

marker). This insertion is in general obtained by the method of gene conversion with the aid of linear deletion cassettes prepared by known methods, as described in Guthrie C. and Fink G.R. eds. Methods in Enzymology, or in Gultner *et al. Nucleic Acid Research*, 1996, 24: 2519-2524.

5 The method of the invention is directed to the isolation of an *S. cerevisiae* strain where the cidal target gene is under the regulation of copper as described above. The preferred copper regulated strains of the invention are those with copper regulated essential proteins which are required for growth and/or viability. In carrying out the method of the invention, an *S. cerevisiae* strain in which expression of a particular gene
10 can be tightly regulated by copper is generated as described above to be used in the methods as described below.

(ii) *Identification of target gene products whose rapid depletion leads to increased sensitivity to known antifungal drugs and candidate antifungal compounds.*
The present invention encompasses methods of identifying target gene products which,
15 when decreased, provide for increased sensitivity to known antifungal drugs or, more importantly, candidate antifungal compounds isolated through various screening methods. Examples of known antifungal agents include amphotericin B and other polyene macrolide compounds like nystatin; flucytosine; ketoconazole, fluconazole, itraconazole and other triazoles. It would be highly advantageous however to isolate new
20 antifungal compounds which attack fungi at one of a number of essential targets.

It has been shown that decreased gene dosage of a drug target results in increased sensitivity of the cell to the drug (Giaever *et al.*, Nature Genetics, 21:(3) 278-283, 1999). Giaever, *et al.* demonstrated this using heterozygous diploid strains of *S. cerevisiae* (one of two gene copies deleted). A similar approach can be used with the

engineered copper strains of the invention. Increasing concentrations of copper in the growth media will lead to decreased gene expression which results in increased sensitivity to compounds that inhibit the activity of the gene product in question. In effect, there is a synergy between copper and certain compounds. Compounds that do not display such synergy are less likely to be hitting the target of interest.

According to the invention, an engineered yeast strain of the invention, where the copper regulated gene has been shown to be an essential target, is used to evaluate and characterize the mechanism of action of such antifungal drugs and candidate antifungal compounds. Alteration of copper ion concentrations in the growth media of Cu-regulated strains leads to a dose-dependent modulation of the expression of the yeast gene product of interest. Altered sensitivity of these strains to a particular compound suggests that the gene is involved in mediating the action of the known antifungal drug, or compound of interest. Comparatively, compounds that do not demonstrate altered sensitivity are unlikely to produce antifungal effects through interaction with the modulated *i.e.* copper regulated gene product. Thus, the engineered yeast strains of the invention provide a tool for screening antifungal compounds in order to characterize the activity and gene product with which the compounds interact. This phenomenon also provides for the establishment and identification of potential synergies between known drugs and newly discovered antifungal compounds.

In one embodiment, such drug sensitivity is analyzed utilizing the copper regulated strains of the invention according to the following non-limiting method. In a single 96 well assay plate, a serial dilution of copper sulfate is created from right to left. On the same plate a serial dilution of compound is created along the opposite axis to give the final compound and copper concentrations shown in Figure 12. Each well is

inoculated with 10^3 cells ml^{-1} . Growth is scored by eye after 48 hours incubation at 37°C .

The patterns of growth expected for each plate are shown in Figures 13a-c. As the copper concentration is increased, the level of target expressed is reduced. Consequently, inhibition of growth should occur at a lower compound concentration (Figure 13, panel

5 a). If the compound does not inhibit the target no effect on growth in the presence of copper will be seen (Figure 13, panel b). A wild type strain should be used as a control to demonstrate that the copper concentration alone is not making the cells more susceptible to the compound. (Figure 13, panel c).

(iii) *Rapid cloning of functionally complementary DNA fragments or*
10 *functionally complementary genes from other organisms, including pathogenic fungi*
such as C. albicans and A. fumigatus. The present invention encompasses methods of rapidly cloning complementary genes from other organisms, particularly other pathogenic fungi, mutant DNA and DNA fragments. According to the invention, the engineered yeast cells of the invention are used to evaluate and characterize the ability of a given
15 gene, mutant DNA or DNA fragment to complement a metal ion-regulated protein. As described above, the engineered yeast cells of the invention provide for the generation of conditional mutant strains where alteration of metal ion concentrations in the growth media of such metal ion-regulated strains leads to a slowing of cell growth and/or cell death by depletion of a particular yeast gene product which is essential for growth and or
20 life of the cells. Transformation of these conditional mutants with cDNA or genomic DNA libraries from other species allows for the selection and identification of functional homologs to the depleted *Sacchromyces* gene product.

This type of analysis can also be used to test which region or regions of a given target protein are essential for function. This is carried out by transforming

the conditional Cu-regulated strains with fragments of the disrupted genes, or DNA isolated from mutants with known disruptions in the gene of interest, in order to determine the regions functionally necessary for complementation.

Complementation analysis utilizing conditional mutants is well known in the field of yeast genetics. Indeed, complementation has been demonstrated for a number of genes. Defects in the following *S. cerevisiae* genes are known to be complemented by homologs from the indicated species:

RHO1	<i>C. albicans</i> , human, drosophila
URA3	<i>C. albicans</i> , <i>S. pombe</i> , <i>C. utilis</i> , mouse
10 CDC68	<i>K. lactis</i>
RPL35	<i>S. pombe</i>
RPB6	<i>S. pombe</i> , human, drosophila
SPT15	<i>C. albicans</i> , <i>S. pombe</i> , <i>A. nidulans</i>
CMD1	<i>S. pombe</i> , <i>K. lactis</i> , <i>Xenopus laevis</i> , chicken

15

Thus, in one embodiment, the invention is directed to a method of rapidly cloning functionally complementary genes from another organism. Complementation analysis may be carried out using a gene or DNA fragment isolated from any organism, including human, mouse, or other mammal; drosophila; and other mycete fungi. Examples of the other mycetes to be analyzed for functionally complementary genes include *Candida albicans*, *Candida stellatoidea*, *Candida tropicalis*, *Candida parapsilosis*, *Candida krusei*, *Candida pseudotropicalis*, *Candida quillermondii*, *Candida glabrata*, *Candida lusitanae* or *Candida rugosa*, or also mycetes of the type *Aspergillus* or *Cryptococcus*, and in particular, for example, *Aspergillus fumigatus*, *Coccidioides immitis*, *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Paracoccidioides brasiliensis* and *Sporothrix schenckii*, or also mycetes of the classes of *Phycomycetes* or *Eumycetes*, in particular the sub-classes of

25

Basidiomycetes, Ascomycetes, Mehliscomycetales (yeast) and *Plectascales, Gymnascales* (fungus of the skin and hair) or of the class of *Hyphomycetes*, in particular the sub-classes *Conidiosporales* and *Thallosporales*, and among these the following species: *Mucor*, *Rhizopus*, *Coccidioides*, *Paracoccidioides* (*Blastomyces*, *brasiliensis*), *Endomyces* 5 (*Blastomyces*), *Aspergillus*, *Menicilium*. (*Scopulariopsis*), *Trichophyton* (*Ctenomyces*), *Epidermophyton*, *Microsporon*, *Piedraia*, *Hormodendron*, *Phialophora*, *Sporotrichon*, *Cryptococcus*, *Candida*, *Geotrichum*, *Trichosporon* or also *Toropsulosis*.

Complementation methods are well known in the art. It is known that given an essential gene in a species, genes which are homologous or have the same 10 function in another species can be identified. The methods known to those of ordinary skill in the art can be used to identify a homolog to a gene studied in another species of mycete (so-called "orthologous" genes) or other organisms genes having the same function as the gene studied. Examples of methods are described in the following works which are hereby incorporated by reference herein:

- 15 - Sambrook et al. 1989, Molecular Cloning, Cold Spring Harbor Laboratory Press.
- Ausubel F. et al. eds. Short Protocols in Molecular Biology, 1995, Wiley.
- Guthrie C. and Fink G.R. eds. Methods in Enzymology, Vol. 194, 1991, 'Guide to Yeast Genetics and Molecular Biology', Academic Press Inc.

Such methods include screening for homology or gene complementation to genomic or 20 cDNA libraries of pathogenic mycetes, or PCR amplification of such library DNA using specific primers selected by virtue of their homology to the nucleotide sequence of interest.

In the present invention, the metal ion-regulated conditional mutants of *Saccharomyces cerevisiae* are used to rapidly determine if a particular gene, gene 25 fragment or DNA can complement and thus be easily selected and cloned. Using the strains of the invention, it is not necessary to carry out homology analysis as described previously. Using the strains of the invention, genes and DNA fragments can be screened rapidly by determining if they provide complementation of an essential gene.

Genomic DNA or cDNA libraries can be prepared by known methods and the polynucleotide fragments obtained are integrated into an expression vector, for example a vector such as pRS423 or its derivatives, which can be used both in the bacterium *E. coli* and in *S. cerevisiae*.

5 In one embodiment, an engineered *S. cerevisiae* strain of the invention in which an identified essential gene has been placed under metal ion regulation is transformed by a representative sample of a DNA or cDNA library corresponding to the organism being studied. Such methods can also be used in the analysis of complementation by a given DNA fragment or mutant DNA. As described above, when
10 the yeast strains of the invention are cultured in the presence of metal ion, the promoter is repressed and the only yeasts that can survive are the ones that carry a recombinant vector containing a sequence which is functionally equivalent to, or complements, the regulated *S. cerevisiae* essential gene.

 Following determination of complementation, the complementing gene
15 or DNA sequence can be further characterized and sequenced by isolating the recombinant vector and sequencing it by known methods.

 This type of study can be performed on various species: the genes which are functionally equivalent or homologous in sequence to an essential gene can be isolated in other mycetes, and in particular in the various mycetes which are pathogenic
20 to humans which include, in particular, mycetes belonging to the classes *Zygomycetes*, *Basidiomycetes*, *Ascomycetes* and *Deuteromycetes*. More particularly, the mycetes will belong to the sub-classes *Candida* spp., in particular *Candida albicans*, *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis* and *Candida krusei*. The mycetes will also belong to the sub-classes *Aspergillus fumigatus*, *Coccidioides immitis*,

Cryptococcus neoformans, *Histoplasma capsulatum*, *Blastomyces dermatidis*,
Paracoccidioides brasiliensis and *Sporothrix schenckii*.

(iv) *Development of libraries of strains, each of which contains a different gene which is either positively or negatively regulated by metal ions.* The present invention encompasses the development of libraries of the metal ion-regulated strains of the invention. Such libraries are useful for identifying targets for antifungal drugs whose mechanism of action is unknown. For example, if stimulation or repression of expression of a particular gene leads to decreased and increased sensitivity, respectively, to a particular drug, then the gene is likely to be involved in mediating the *in vivo* action of the drug. Evaluating the effect of modulation of the gene product, by the presence or absence of metal ion, on cell physiology leads to understanding the physiological role of the gene product. Further, comparison of phenotypes associated with cells depleted of certain gene products should yield similar phenotypes in that compounds inhibit that gene product. Large strain libraries can easily be created since the entire *S. cerevisiae* genome has been sequenced and all open reading frames identified (Goffeau et al., *Science* 274:563, 1996; Goffeau et al., *Nature* 387:5, 1997). The majority of these open reading frames and corresponding encoded proteins have been characterized (Costanzo et al., *Nucleic Acids Res.* 28:73, 2000; Ball et al., *Nucleic Acids Res.* 28:77, 2000; Mewes et al., *Nucleic Acids Res.* 28:37, 2000)

(v) *Development of high-throughput screening methodologies to detect antifungal compounds.* The present invention also encompasses methods wherein the engineered yeast cells of the invention are used to evaluate and characterize compounds as potential antifungal compounds. Alteration of the concentration of metal ions leads to a dose-dependent modulation of the expression of the regulated yeast gene

product. Altered sensitivity of these cells to a particular compound, suggests that the controlled gene product is likely to be involved in mediating the action of the compound and lead to the determination of that compound as a candidate inhibitor.

Such compounds may be found in, for example, natural product libraries, fermentation libraries (encompassing plants and microorganisms), combinatorial libraries, compound files, and synthetic compound libraries. For example, synthetic compound libraries are commercially available from Maybridge Chemical Co. (Trevillet, Cornwall, UK), Comgenex (Princeton, NJ), Brandon Associates (Merrimack, NH), and Microsource (New Milford, CT). A rare chemical library is available from Aldrich Chemical Company, Inc. (Milwaukee, WI). Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available from, for example, Pan Laboratories (Bothell, WA) or MycoSearch (NC), or are readily producible. Additionally, natural and synthetically produced libraries and compounds are readily modified through conventional chemical, physical, and biochemical means (Blondelle et al., *TibTech* 14:60, 1996).

Thus, in a further embodiment of the invention, following the determination that a given *Saccharomyces cerevisiae* strain of the invention contains a metal ion-regulated cidal target, the strain may be used to isolate candidate inhibitors of fungal growth and/or infection. In carrying out the screening methods of the invention, which involve screening a plurality of candidate inhibitor compounds, *i.e.* candidate antifungal compounds, the plurality of candidate antifungal compounds may be screened together in a single assay or individually using multiple simultaneous individual detecting steps. The methods used to determine altered sensitivity to candidate antifungal compounds as described above are used in carrying out the screening of the plurality of candidates.

As noted above, a "candidate inhibitor," as used herein, is any compound

with a potential to inhibit the growth and viability of *Saccharomyces cerevisiae*, or other fungi. The methods of the invention are directed to the identification of candidate inhibitor compounds in a primary screen against the *Saccharomyces cerevisiae* metal ion-regulated strains of the invention.

5 Candidate inhibitors are screened by measuring susceptibility of the *Saccharomyces cerevisiae* strains of the invention where an essential gene is under the control of metal ion. According to the methods of the invention, a plurality of candidate inhibitors are screened utilizing a given strain of the invention to determine if the cells are killed or grow slower in a lower concentration of metal ion. If a lower concentration
10 of metal ion is required to kill or slow growth, then the compound being screened is characterized as a potential antifungal.

Candidate Inhibitor Preparation:

 Stock solutions and concentrations tested will vary from compound to
15 compound. In one non-limiting embodiment, stock solutions of 12.8 mg/ml in DMSO (Sigma D-8779) should be prepared. This will allow for a 128 ug/ml starting test concentration containing 1% DMSO. Stock solutions should be stored at -20°C and dilutions for antifungal testing should be freshly prepared before each assay.

 For compounds of unknown activity or ones with MIC values of >4 ug/ml,
20 a range of concentrations from 128 ug/ml to 0.125 ug/ml should be used. More active compounds, such as Amphotericin B (Sigma A2411) and Itraconazole (Research Diagnostics Inc. cat# 30.211.44), require a lower range of concentrations (16 ug/ml to 0.016 ug/ml). Stock solutions of Amphotericin B and Itraconazole should be prepared at 1.6 mg/ml in DMSO. Amphotericin B is sold as a powder that is approximately 80%
25 Amphotericin B. Stock solutions should be made accordingly (2.0 mg of powder for a 1 ml solution of 1.6 mg/ml Amphotericin B).

Stock solutions of control compounds (1.6 mg/ml, Amphotericin B or Itraconazole) are initially diluted in medium to a concentration of 32 ug/ml while stock solutions of test compounds (typically 12.8 mg/ml) are diluted to 256 ug/ml. Both of these (control and test compounds) represent 1:50 dilutions. If a stock solution of a test compound is not at 12.8 mg/ml, the appropriate dilution must be calculated. Serial dilutions will be produced (see below) using these initial dilutions. Addition of cells to compound will produce an additional two-fold dilution.

Natural product extracts are tested at concentrations ranging from 200 to 204,800 fold dilution of the extract based upon the initial culture volume. The extract should first be diluted 100 fold then serial dilutions produced as directed below.

* * * * *

Compounds that interact with the target gene of interest with have an effect on the concentration of metal ion required to kill or slow the growth of the strain of interest. Such compounds are considered for further development.

(vi) *Phenocopy control for drug treated cells.* The present invention also encompasses methods of determining the phenotype generated by a particular antifungal agent. Phenotypes associated with cells that are depleted of a specific gene product, should produce a similar phenotype to that of cells treated with a compound that specifically inhibits that protein as its target. If a metal ion repressed cell produces the same phenotype as a cell treated with a particular antifungal drug, it would suggest that the compound is producing its effect through interaction with the gene or gene product being evaluated. Indeed, if the phenotype of a compound treated strain is similar to or the same as the phenotype of the target gene, such compound can be associated with inactivation of the target gene or protein.

Phenotypes associated with decreased cell viability and/or growth can fall into many categories. Non-limiting examples of such categories may include: absence of DNA synthesis, absence of RNA synthesis, absence of transfer of RNA, absence of tRNA, absence of cell division, absence of cell cycling, absence of growth, and inability to make membrane. Non-limiting examples of essential cellular functions and associated genes are growth (BOS1); viability (RPL31); cell cycle control (CDC23); cell wall maintenance (GPL3); DNA synthesis (POL30); nuclear-cytoplasmic transport (NPL6); tRNA synthesis (RPC34); rRNA synthesis (RRN3); mRNA synthesis (SRB4); protein synthesis (NIP1); and RNA splicing (PRP21). References for some of these examples, as well as other examples of cellular functions and known mutations in *S. cerevisiae* are provided as follows:

*** Protein transport from the endoplasmic reticulum (ER) to the Golgi complex**

BOS1 mutants

Wuestehube LJ, Duden R, Eun A, Hamamoto S, Korn P, Ram R, Schekman R. New mutants of *Saccharomyces cerevisiae* affected in the transport of proteins from the endoplasmic reticulum to the Golgi complex.

Genetics 1996 Feb;142(2):393-406

*** DNA replication and repair**

POL30 mutants

Merrill BJ, Holm C. The RAD52 recombinational repair pathway is essential in pol30 (PCNA) mutants that accumulate small

single-stranded DNA fragments during DNA synthesis. Genetics 1998 Feb;148(2):611-24

*** Protein translation**

DED1 mutants

Chuang RY, Weaver PL, Liu Z, Chang TH. Requirement of the DEAD-Box protein ded1p for messenger RNA translation. Science 1997 Mar 7;275(5305):1468-71.

*** mRNA splicing**

PRP21 mutants

- Arenas JE, Abelson JN. The *Saccharomyces cerevisiae* PRP21 gene product is an integral component of the prespliceosome. *Proc Natl Acad Sci U S A* 1993 Jul 15;90(14):6771-6775.

*** tRNA splicing**

TRL1 mutants

- Phizicky EM, Consaul SA, Nehrke KW, Abelson J. Yeast tRNA ligase mutants are nonviable and accumulate tRNA splicing intermediates. *J Biol Chem* 1992 Mar 5;267(7):4577-82

*** rRNA synthesis**

RRN3 mutants

- Nogi Y, Vu L, Nomura M. An approach for isolation of mutants defective in 35S ribosomal RNA synthesis in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci U S A* 1991 Aug 15;88(16):7026-30

*** Long chain fatty acid synthesis**

ACC1 mutants

- Schneider R, Hitomi M, Ivessa AS, Fasch EV, Kohlwein SD, Tartakoff AM. A yeast acetyl coenzyme A carboxylase mutant links very-long-chain fatty acid synthesis to the structure and function of the nuclear membrane-pore complex. *Mol Cell Biol* 1996 Dec;16(12):7161-72

*** Protein N-glycosylation**

ALG1mutants

- Benton BK, Plump SD, Roos J, Lennarz WJ, Cross FR. Over-expression of *S. cerevisiae* G1 cyclins restores the viability of *alg1* N-glycosylation mutants. *Curr Genet* 1996 Jan;29(2):106-13

*** Sphingolipid synthesis**

AUR1 mutants

Nagiec MM, Nagiec EE, Baltisberger JA, Wells GB, Lester RL, Dickson RC. Sphingolipid synthesis as a target for antifungal drugs.

- 5 Complementation of the inositol phosphorylceramide synthase defect in a mutant strain of *Saccharomyces cerevisiae* by the AUR1 gene. J Biol Chem 1997 Apr 11;272(15):9809-17

* tRNA nucleotidyltransferase

CCA1 mutants

- 10 Chen JY, Joyce PB, Wolfe CL, Steffen MC, Martin NC. Cytoplasmic and mitochondrial tRNA nucleotidyltransferase activities are derived from the same gene in the yeast *Saccharomyces cerevisiae*. J Biol Chem 1992 Jul 25;267(21):14879-83

15 * Ergosterol biosynthesis

ERG11 mutants

- Trocha PJ, Jasne SJ, Sprinson DB. Yeast mutants blocked in removing the lanosterol at C-14. Separation of sterols by high-pressure liquid chromatography. Biochemistry 1977 Oct 18;16(21):4721-6

20

* Polyadenylation

FIP1 mutants

- Preker PJ, Lingner J, Minvielle-Sebastia L, Keller W. The FIP1 gene encodes a component of a yeast pre-mRNA polyadenylation factor that
25 directly interacts with poly(A) polymerase. Cell 1995 May 5;81(3):379-89.

* Nuclear export of RNA

GLE1 mutants

- 30 Murphy R, Wente SR. An RNA-export mediator with an essential nuclear export signal. Nature 1996 Sep 26;383(6598):357-60

* rRNA processing

LCP5 mutants

Wiederkehr T, Pretot RF, Minvielle-Sebastia L. Synthetic lethal interactions with conditional poly(A) polymerase alleles identify
5 LCP5, a gene involved in 18S rRNA maturation. RNA 1998
Nov;4(11):1357-72

With the emergence of high throughput screening ("HTS") methodologies for identifying candidate antifungal compounds, more and more New Chemical Identities
10 ("NCI") have been identified. There is thus an urgent need to establish a systematic method to study the mechanism of action of those NCIs in a high throughput fashion.

In one embodiment of the invention, in-cell macromolecular labeling is utilized to analyze the target of action of those candidate compounds with regard to different the biosynthetic pathways. A non-limiting example of one method is
15 summarized briefly as follows: Radio-labeled building-blocks of different macromolecules are incubated with yeast and the incorporation of these radio-labeled building-blocks is measured either in the presence or in the absence of the compounds of interest. The compounds that inhibit a specific macromolecular synthetic pathway can be quickly identified and further characterized.

20 This method is especially powerful when applied to an *S. cerevisiae* metal ion regulated strain of the invention that can transiently reduce the expression of a target gene in the presence of metal ion. When the target gene is transiently knocked out, the synthetic pathway that involves that gene will be affected immediately and the incorporation of the building-blocks will be reduced. This effect can be used to
25 characterize particular phenotypes associated with the target gene of interest. Analysis of antifungal compounds using the macormolecular assay described above and comparing the results to those obtained with the regulatable strains of the invention allows for the rapid determination of a phenotype for the candidate antifungal. Information about

expected phenotypes for antifungal compounds is thus further augmented when analyzing a compound that specifically inhibits the function of a given target gene.

One example of the methodologies used in these analyses of antifungal compounds is as follows. Similar methods may be employed when analyzing the regulated yeast strains of the invention.

Preparation

Culture:

Yeast is grown at 37°C overnight in a rotary bath in YNB medium with 0.05% glucose, 0.05% Difco Casamino acids, 0.1mg/L adenine and 0.14 mg/L N-acetylglucosamine. 5 ml of overnight culture is added to 25 ml of the same media until the O.D. reaches 0.5.

Radiolabeling:

N-Acetyl-D(1-3H)glucosamine	Amersham: TRK376, 9.9 Ci/mmol, 1 mCi/ml
D-(5-3H)Glucose	Amersham: TRK290, 17.4 Ci/mmol, 1 mCi/ml
L(4,5-3H)Leucine	Amersham: TRK510, 148Ci/mmol, 1mCi/ml
(2-3H)Adenine	Amersham: TRK311, 21 Ci/mmol, 1mCi/ml

20

Five different tubes containing the following supplements are setup:

1. DNA: 40 µl radiolabeled Adenine + 8 µl unlabeled Adenine.
2. Glucan: 40 µl radiolabeled glucose
3. Chitin: 40 µl radiolabeled N-acetylglucosamine + 4 µl unlabeled N-acetylglucosamine
4. Protein: 16 µl radiolabeled Leucine
5. RNA: 10 µl radiolabeled Adenine + 10 µl unlabeled Adenine.

25

Reaction-stop:

Setup 96-well plate for different stop-reaction:

1. Protein synthesis can be stopped in the plate with 75 μ l of 10% TCA in each well.
 - 5 2. RNA synthesis can be stopped in the plate with 75 μ l of 10% TCA in each well too but has to be in different plate with protein.
 3. DNA, Glucan and chitin synthesis can be stopped in the same plate with 75 μ l of 12% KOH.
 - 10 Holes should be drilled on each corner of the plates for better floating on water bath later on.
- Put the plate on ice to make reaction stop better.

Compounds:

- 15 For each macromolecular synthesis, three different concentrations (including 0 μ g/ml) of the testing compound are setup in three different tubes.

Reaction:

Each time point is be duplicated.

- 20 Four mls of the yeast culture are added to each different tube and timer is started to click. Take 75 μ l of each individual radiolabeling as background.

After incubation for 5 minutes, 1.2 ml of each reaction is added to the tubes containing testing compounds and 75 μ l of reaction is immediately taken to stop solution.

25

Repeat stop-reaction at 15, 20, 25, 35, 45, 55 minutes.

Post-reaction:

Precipitation:

Plates measuring DNA, glucan and chitin synthesis are heated for 90 minutes at 80°C. Then add 24 µl of 37% HCl and 19.3 µl of cold 50% TCA, incubated at 4°C for 30 minutes.

5

Plates measuring RNA synthesis are incubated at 4°C for 30 minutes.

Plates measuring protein synthesis are heated for 15 minutes at 80°C and then incubated at 4°C for 30 minutes.

10

Filtration:

Plates are filtered using GF-A plates usually machine will give better results. Wash twice with 200 µl 10% TCA and incubated at 65°C to let them dry.

15

Count:

Add 20 µl of scintillation fluid and counted

References:

For DNA, RNA and Protein synthesis:

20

Landini P, Corti E, Goldstein BP, Denaro M. Mechanism of action of purpuromycin. *Biochem J.* 1992 Jun 15;284 (Pt 3):935.

For Glucan synthesis:

25

Baguley BC, Rommele G, Gruner J, Wehrli W. Papulacandin B: an inhibitor of glucan synthesis in yeast spheroplasts. *Eur J Biochem.* 1979 Jul;97(2):345-51.

For RNA and protein:

Onishi JC, Milligan JA, Basilio A, Bergstrom J, Curotto J, Huang L,

Meinz M, Nallin-Omstead M, Pelaez F, Rew D, Salvatore, M, Thompson J, Vicente F, Kurtz MB. Antimicrobial activity of viridiofungins. *J Antibiot* (Tokyo). 1997 Apr;50(4):334-8.

5 The following examples are intended as non-limiting illustrations of the present invention.

Example 1: Construction of a Copper-Tolerant Yeast Strain Containing a Copper-Regulated ROX1 Gene

10 The following experiments were performed to: (i) examine the copper sensitivity of different yeast strains; and, (ii) to create a copper-dependent yeast strain containing a copper-regulatable ROX1 gene.

 The copper sensitivity of yeast strain CTY145 was compared with that of ZMY60 by growing each strain in the presence of increasing amounts of added copper and measuring cell number at increasing times. After 16 hours of growth, the highest
15 concentration of copper at which the log-phase growth rate of ZMY60 was maintained unaffected was 250uM. (Figure 1). By contrast, the highest concentration of copper at which the growth rate of CTY145 was maintained was 1 mM. Thus, CTY145 is at least four-fold more tolerant to copper than ZMY60.

20 A strain based on CTY145 that contains a copper inducible ROX1 was constructed as follows. Using a conventional lithium acetate/polyethylene glycol technique, CTY145 was transformed with approximately 0.1 ug of plasmid pZM195 (Figures 4A-D), which was linearized with the restriction enzyme AflII. The plasmid contains a metal responsive element comprising HIS3 promoter sequences fused to
25 ACE1.

Integration of the plasmid into the genome was monitored by the ability of the cells to grow on -URA plates (growth of the yeast strains in this medium requires the functional URA3 gene contained on the plasmid). After 48 hours at 30°C, the transformants were inoculated onto fresh -URA plates and regrown for an additional 48
5 hours at 30°C. Well spaced individual colonies were picked and inoculated to 5 ml YPD media, grown overnight at 30°C, and 5 µl of the culture was inoculated into 5-FOA plates. In the presence of a functional URA3 gene, 5-FOA is converted to a toxin which kills cells. Therefore, the only cells that survive are those that have lost the URA3 gene by recombination.

10 Integration of ZM195 into the genome could occur in any of the following ways: (1) The original integration could occur non-specifically. In this case, the 5-FOA-induced deletion of URA3 would have no effect. (2) The original incorporation could occur specifically, and the subsequent 5-FOA induced deletion would result in a return to the original promoter sequence. (3) The original integration could occur specifically,
15 and the subsequent 5-FOA induced deletion of URA3 could lead to the correct insertion of the copper-inducible promoter directly upstream of the ROX1 open-reading frame (ORF).

To detect the correct promoter insertion, three PCR primers were designed: ROX-A (5'-TCACACAAAAGAACGCAG-3') (SEQ ID NO:4), corresponding
20 to a sequence from the region of the original promoter immediately 5' to the first ATG of the ORF; ROX-B (5'-GATGACAGCTGTGGTAGG-3') (SEQ ID NO:5), the reverse complement of a sequence in the ORF of ROX1 which is not present in ZM195; and ROX-C (5'-TCTTGCCATATGGATCTG-3') (SEQ ID NO:6), a sequence internal to the copper inducible promoter. For possibilities 1 and 2 above, PCR amplification of

genomic DNA with ROX-A and ROX-B would lead to a 601 base pair (bp) product, and PCR amplification with ROX-B and ROX-C would yield no product. For possibility 3, the correct insertion, PCR using ROX-A and ROX-B would yield a 2628 bp product, and PCR with ROX-B and ROX-C would yield a 785 bp product. PCR analysis identified
5 a strain that had undergone the correct rearrangements. This strain was designated CUY101.

To bring the UBR1 gene under the control of the copper inducible promoter, HIS3-ACE1, the above-described procedure was repeated using CUY101 as the starting strain. The ZM197 plasmid (Figures 5A-D) that had been linearized by
10 digestion with the restriction enzyme AatII was introduced into the cells. To identify cells in which the correct promoter insertion had occurred, three PCR test primers were designed: UBR-A (5'-ATCTTCGGACAAAGGCAG-3') (SEQ ID NO: 7); UBR-B (5'-GTGTAATTTTCGGGATCG-3') (SEQ ID NO:8) and ROX-C (5'-TCTTGCCATATGGATCTG-3') (SEQ ID NO:9). PCR analysis is used to identify one
15 culture which has undergone the correct rearrangements. This strain is designated CUY103.

Example 2: Construction of a Yeast Strain Containing A Deletion of SLF1

In practicing the present invention, it is preferred that copper regulation
20 of expression be maintained over a relatively long time period, i.e., for several days. The transient effect of copper in wildtype yeast is due at least in part to the fact that yeast cells are able to biomineralize copper. Thus, over time wild-type yeast cells deplete the medium of copper and the effect on expression is lost. If biomineralization activity is ablated, then the extracellular copper levels should remain nearly constant over time.

25 The only known gene in the yeast copper ion biomineralization pathway is the SLF1 gene. Inactivation of the SLF1 gene has been shown to result in cells which are slightly more sensitive to copper but are unable to efficiently deplete copper from the

media (Yu et al., *Mol. Cell Biol.* **16**:2464, 1996). Therefore, the SLF1 gene in the yeast strains described in Example 1 above was inactivated.

A construct was created for a two-step knockout of the SLF1 open reading frame. Primers SLF-E (5'-GCGCTGCAGGTCGACTTAGCAGGCAGTTTGAAC-3') (SEQ ID NO:10) and SLF-F (5'-GCGCTGCAGGCATGCACTCCTTTCCAATTGTGC-3') (SEQ ID NO:11) were used to amplify the 3'- untranslated region of SLF1 using genomic DNA as template. The Sall/SphI fragment of the PCR product was subcloned into Sall/SphI-digested pUC19 plasmid (Genbank accession no. M77789). This recombinant plasmid was designated pSLF3'. Similarly, primers SLF-G (5'-GCGAGCTCGGTACCCCATACCCCTAACTCTAG-3')(SEQ ID NO: 12) and SLF-H (5'-GCGGATCCCGGGGCTCTCTCGTTTATTTAACG-3')(SEQ ID NO:13) were used to amplify the 5'- untranslated region of SLF1, and the SacI/BamHI or KpnI/BamHI fragment was cloned into SacI/BamHI or KpnI/BamHI digested pSLF3' to produce pSLF3'5'. The 5.5 kb BamHI/XbaI insert of pDJ20, which contains the yeast URA3 gene and bacterial kanamycin resistance gene flanked by a direct repeat of the *Salmonella* HisG sequence, was subcloned into XbaI/BamHI digested pSLF3'5' to create pSLFKO. Plasmid pDJ20 is derived from the plasmid pSP72 (Promega, Madison, WI) into the BamHI site of which has been cloned the approximately 5.5. kb insert consisting of the following elements:

20 | hisG | URA3 | kanamycin resistance | hisG |

The hisG elements are present as a tandem repeat. Plasmids containing this element can be transformed into bacteria for amplification; selection with kanamycin helps to avoid unwanted recombination between the two hisG regions in bacteria which would result in the loss of the *S. cerevisiae* URA3 gene. The hisG, URA3 and kanamycin genes are

well-known in the art and can be assembled in this order by conventional techniques in molecular biology, and do not need to be obtained from plasmid pDJ20.

This plasmid (pSLFKO) was digested with SphI and EcoRI and transformed into strains ZMY60, CTY145, CUY101, and CUY103 using a conventional
5 lithium acetate/polyethylene glycol technique, as described above in the Methods section. Integration of the plasmid into the genome of each yeast strain was monitored by the ability of the strain to grow on (-)URA plates. After 48 hours at 30°C, the transformants were inoculated onto fresh (-)URA plates and regrown for an additional 48 hours at 30°C. Well spaced individual colonies were inoculated into 5 ml YPD media, grown overnight
10 at 30 °C, and 5 µl of the culture was inoculated onto 5-FOA plates.

In the presence of a functional URA3 gene, 5-FOA is converted to a toxin which kills cells. Therefore, the only cells that survive are those which have lost the URA3 gene by recombination. Either of the following could occur: (1) Non-specific integration of the linear DNA containing the 5'-HisG-URA3-kanR-HisG-3'NTS fragment
15 could occur, followed by deletion of the region between the HisG repeats; this would result in a 5'NTS-HisG-3'NTS integration at some random spot. (2) Alternatively, specific integration of the linear DNA containing the 5'NTS-HisG-URA3-kanR-HisG-3'NTS sequence could occur, followed by deletion, which would result in a deletion/insertion in which the entire ORF of SLF1 has been deleted and a single copy
20 of the HisG element has been left in its place.

To confirm that the correct genetic alteration occurred, PCR was performed using the following sets of primers: (i) HISGCH (5'-GATTTGGTCTCTACCGGC-3') (SEQ ID NO: 14) and SLF-D (5'-GACAGTATCGTAATTACG-3') (SEQ ID NO: 15); and (ii) a primer comprising the
25 reverse complement of primer HISGCH and SLF-D as above. Alternatively, PCR with SLF-A (5'-CTAACTCTAGCTGCATTG-3') (SEQ ID NO: 24) (or SLF-G) and SLF-D (or SLF-F) could be used to produce a diagnostic shift in product length after PCR. The SLF1 deleted version of CTY145 is designated CUY104; the SLF1 deleted version of

CUY101 is designated CUY105; the SLF1 deleted version of CUY103 is designated CUY106; and, the SLF1 deleted version of ZMY60 is designated CUY107.

Cultures of yeast strains CTY145, CUY101, CUY103, CUY104, CUY105, and CUY106 were cultured in 5 ml of complete synthetic media (CSM) supplemented with 500 mM CuSO₄ at 30 °C in a rollerdrum apparatus at a speed of approximately 60 revolutions per minute. At 24 and 48 hours, the cultures were pelleted and resuspended in fresh CSM media containing 500 mM CuSO₄. After 96 hours of incubation, cells were collected onto filter paper and the supernatant was removed by suction through the filter paper. Biom mineralization is inferred by the presence of a darkened cell pellet, indicating the biom mineralization of the soluble copper to copper sulfide (CuS) which has been shown to be deposited on the cell surface. Strains CTY145, CUY101, and CUY103 (A, B, and C, respectively in Figure 9) contain the wild-type SLF1 gene, as demonstrated by their dark color. Strains CUY104, CUY105, and CUY106, in which the SLF1 gene has been deleted, show considerably lighter coloration after collection on filter paper, indicating an ablation of copper biom mineralization activity.

Single colonies of each yeast strain described above were picked from YPD plates and grown overnight in YPD media at 30 °C with shaking. Cultures were diluted to an absorbance at 600 nm of 0.02 in CSM media containing various concentrations of CuSO₄. Cultures were grown for 24 hours at 30 °C in a rollerdrum apparatus at approximately 60 revolutions per minute. The absorbance of each culture at 600 nm, which is a measure of cell density (i.e., the number of cells in a culture) was measured. The results of the cell density assays are shown in Figure 10, and is expressed as a percentage of the cell density achieved in cells with no added copper sulfate.

Example 3: Method for Stable Replacement of the Promoter Element of Any Gene of Interest with a Copper-Inducible Promoter

The following procedures were performed to stably replace the promoter
5 element of any yeast gene of interest. The strategy is designed to avoid: (1) the use of
URA3 as a selectable marker, which precludes its use in future selection procedures; (2)
the requirement for a naturally occurring unique restriction site in the coding sequence
of the subject gene; (3) the need for multiple subclonings; and (4) the need for constant
maintenance of URA3 selection in order to prevent loss of the inserted sequence, which
10 would result in restoration of the original promoter elements.

A single or double-PCR strategy was devised. Instead of a single
crossover event, the method requires that a double crossover occur in order to achieve
integration into the yeast genome. Although a double crossover event is less likely to
occur, once it has occurred the resulting transformed haploid yeast strain does not have
15 to be maintained under selection. HIS3 is used as a marker to avoid using URA3
unnecessarily; in addition, the HIS3 gene is relatively short and therefore comparatively
easy to amplify.

A plasmid designated pCU3 was constructed which contains a functional
HIS3 gene (including upstream sequences) in inverted orientation to, and upstream from,
20 the ANB1 promoter. The ANB1 promoter was fused upstream (i.e., 5' to) sequences
encoding ubiquitin tag elements. For this purpose, the BamHI/PstI fragment of
pUC8-Sc2676 was subcloned into pUC19. Then, the EcoRI-KpnI fragment of ZM168,
which contains the ANB1 promoter and the ubiquitin tag regions, was subcloned into the
plasmid.

Primers were designed for use in either a one-step or two-step PCR strategy. (Figures 2A-C).

One-Step PCR Strategy: 100-mer oligonucleotides were synthesized. Primer 1 contains 80 bp of target sequence from the gene of interest, which is obtained
5 from knowledge of the DNA sequence immediately 5' to the protein-coding sequence of the gene, together with 20 bp of plasmid sequence. A second set of primers comprising sequences from the non-coding strand is synthesized. These oligonucleotides, which are designated either Primer 2b (5'-CCAGACTACGCTTCGATATCG-3') (SEQ ID NO: 16) ("Tag") or Primer 2a (5'-CACAATAAAACATCGATATT-3') (SEQ ID NO: 17) ("NO
10 TAG"), are then fused to 18-20 bp of the protein-coding sequence of a gene of interest, beginning with the initiator ATG codon. In this case, "Tag" refers to the presence or absence of the ubiquitin tag.

Primer pairs 1 and 2a or 1 and 2b are used to amplify a DNA fragment from pCU3, producing a fragment consisting of genomic 5'NTS (non-translated
15 sequence) followed by HIS3 in an inverted orientation, ANB1 promoter, and either a fragment of the ORF or a tag sequence fused in frame to a fragment of the ORF. Transformation of haploid yeast strains with these sequences, followed by double crossover, leads to integration into the genome. This results in insertion of HIS3, the ANB1 promoter and (in some cases) the tag sequence 5' to the gene of interest.

20 Using this approach, no DNA sequence is lost and no sequence is duplicated, thereby considerably lessening the likelihood that the inserted sequence will be spontaneously deleted. After selection with HIS3, the presence and orientation of the insert is confirmed using PCR. Because the integration requires a double crossover, selection by HIS selection should not be required to maintain the genotype.

25 *Two-Step PCR Strategy:* For the two step strategy, Primer 2a or 2b is fused to 18-20 bp of the ORF of a gene of interest, beginning with the initial ATG of the

ORF. Either primer, and a second primer comprising 18-20 nucleotides that is the reverse complement of a sequence 400-1000 bp downstream, are used to amplify a 400-1000 bp fragment that is the reverse complement of the ORF and has a 3' tag complementary to the sequence in pCU3 such that the sequence is fused in frame to the tags or is fused in frame in place of the tags.

The fragment corresponding to the opposite end is produced by fusing a primer designated "Universal HIS3-2STEP" (5'-CAGGCATGCAAGCTTGGCGT-3') (SEQ ID NO:18) to an 18- to 20-mer representing the reverse complement immediately 5' of the starting ATG of the ORF. This fragment is used in conjunction with a primer identical to 18-20 nucleotides comprising a sequence 400-1000 bp 5' to the starting ATG to amplify a fragment whose 3' end is complementary to the 3' end of the HIS3 gene in pCU3.

The two fragments are then used to amplify pCU3, producing a fragment comprising a length of genomic 5'NTS followed by HIS3 in inverted orientation, ANB1 promoter and either a length of the ORF or a tag sequence fused in frame to a length of the ORF. Transformation with this sequence and double crossover leads to integration into the genome, which results in the insertion of HIS3, the ANB1 promoter and (in some cases) the tag sequence. Using this approach, no DNA sequence is lost and no sequence is duplicated, thereby greatly lessening the likelihood of spontaneous deletion. After selection with HIS3, the presence and orientation of the insert is confirmed with PCR. Because the integration requires a double crossover, HIS selection should not be required to maintain the genotype.

Example 4: Alternative Method for Stable Replacement of the Promoter Element of Any Gene of Interest with a Copper-Inducible Promoter

PCR primers are designed to amplify sequences of the target gene to result in a “shuffled gene” arrangement (Figure 6) in the vector pCU19Srf (Figure 8). The PCR primers that can be used will vary from 18 to 36 nucleotides in length, and will preferentially have a GC content of at least 50 percent. When primers, which are 18 to 36 nucleotides in length have a low GC content, the primers can be made 3 to 6 nucleotides longer than those with at least a 50% GC content.

PCR primers are dissolved in sterile water at a concentration of 1 mg/ml. Genomic DNA from *S. cerevisiae* is diluted to various concentrations in sterile water. Taq DNA polymerase (Promega Biotech, Madison, WI) is typically used for the primary PCR reaction, but other thermostable polymerases, such as Vent polymerase (New England Biolabs, Beverly, MA) or Pfu polymerase (Stratagene, Carlsbad, CA) can also be used.

The shuffled gene of interest is generated by performing two primary PCR reactions. In one, a portion of the target gene which starts about 400 base pairs upstream of the ATG start codon and ends just upstream of the ATG start codon is amplified. In the second primary PCR reaction, a portion of the target gene that starts at or that is just upstream of the ATG start codon and that ends about 400 base pairs downstream of the ATG start codon is amplified. A typical primary PCR reaction will include 10 µl of 10X Taq buffer, 10 µl of 25 mM deoxynucleoside triphosphates, 10 µl of 25 mM MgCl₂, 1 µl of *S. cerevisiae* genomic DNA, and 1 µl of primer pairs (Primers 1 and 2, or primers 3 and 4) at 100 µg/ml, and 66 µl of sterile water.

Typically, primer 1 will consist (from the 5' to the 3' end) of a seven nucleotide sequence, then a NotI restriction endonuclease cleavage site (or a restriction site susceptible to cleavage by another rare-cutting enzyme), followed by 10 or 11 nucleotides which are identical to the top strand of the gene of interest about 400 base pairs upstream of the ATG start codon of the target gene. The seven nucleotides at the 5' end of primer 1 are complementary to the 7 nucleotides immediately 3' of the NotI site in primer 4.

Primer 2 will have the sequence of the bottom strand of the gene of interest, just upstream of the ATG start codon, and will comprise about 18 to about 21 nucleotides.

Primer 3 will have the sequence of the top strand of the target gene either at or very close to the ATG start codon.

Primer 4 will consist of a seven nucleotide sequence at the 5' end, followed by a NotI restriction endonuclease cleavage site (or a restriction site susceptible to cleavage by another rare-cutting enzyme), followed by 10 or 11 nucleotides which are identical to the bottom strand of the gene of interest about 400 base pairs downstream of the ATG start codon. The seven nucleotides at the 5' end of primer 4 are complementary to the 7 nucleotides immediately 3' of the NotI site in primer 1.

Typically, the reaction is initiated by heating the reaction mixtures to 94 °C for 3 to 5 minutes, followed by the addition of 5 units of Taq DNA polymerase. The reaction is then thermocycled reaction mixture through 1 minute at 94 °C, 1 minute at 50 °C, and 2 minutes at 72 °C for 30 cycles, after which the temperature is reduced to 4 °C. The PCR products are run on an agarose gel to determine the conditions that produced a fragment of the appropriate size, typically about 400 base pairs. Modifications of

various parameters of the method in order to optimize reaction conditions, such as altering annealing temperatures, salt concentrations, and the like are within the skill of the ordinarily skilled worker.

The secondary PCR reaction uses the primary PCR reaction products as
5 DNA templates. The one end of each of the two primary PCR products are homologous, and when melted, will anneal to each other over a stretch of about 21 base pairs. The primers used for the secondary PCR reaction are primers 2 and 3 used in the primary PCR reactions. Use of these primers will anneal to the ends of the annealed template DNA and allow the PCR reaction to produce a shuffled gene reaction product.

10 A typical secondary PCR reaction will include 10 μ l of 10X Pfu buffer, 10 μ l of 25 mM deoxynucleoside triphosphates, 1 μ l of primer pairs (i.e., primers 2 and 3 from the primary PCR reaction) at a concentration of 100 μ g/ml, 77 μ l of sterile water, various dilutions of the primary PCR products, and 1 μ l of Pfu polymerase, comprising 2.5 activity units. The PCR conditions are identical to those to be used in the primary
15 reactions, except that the initial heating of the tubes to 94 °C for 3 to 5 minutes is omitted.

The secondary PCR product is electrophoresed through an agarose gel, according to methods well-known in the art, and the appropriate band (of about 800 to 900 base pairs) is cut out. The DNA is then extracted from the gel using, e.g., the Gene
20 Clean kit (Bio101, Vista, CA). The extracted, purified DNA is then used for ligation into the vector pCu19Srf (Figures 8).

To perform the ligation, the PCR-Script kit from Stratagene (La Jolla, CA) is used. pCu19Srf is cut with SrfI restriction endonuclease. The ligation mix contains 100 ng of SrfI cut vector DNA, 1 μ l of 10X PCR Script buffer, 0.5 μ l ATP, 4 to 6 μ l of

insert DNA, containing from 100 to 500 ng, 1 μ l of T4 DNA ligase, and 1 μ l of SrfI. All reagents are provided in the PCR Script kit except for the DNA. However, such reagents are well-known and commercially available from other sources. Three μ ls of the ligation reaction is transformed into competent DH5 α cells, which can be obtained from
5 Gibco/BRL (Rockville, MD), and the cells are plated on LB medium with 100 μ g/ml ampicillin. The plates are incubated at 37 °C for 16 to 18 hours. Single colonies are chosen for restriction enzyme digestion and analysis of the resulting fragments to identify a clone which contains the insert in the proper orientation. A colony that is identified as containing a plasmid with the insert in the proper orientation is selected and is amplified
10 by culturing. The insert-containing plasmid is purified from the bacterial host using the DNA isolation procedure of the Qiagen DNA preparation kit (Qiagen, Hilden, Germany) or other well-known methods for plasmid purification. The purified DNA is digested with NotI restriction endonuclease (or another rare-cutting endonuclease whose restriction site has been engineered into the shuffled gene). The endonuclease is
15 inactivated by heating for 20 minutes at 65 °C. The purified, cut DNA is then used to transform *S. cerevisiae*.

Strain CUY106 (Ace-ROX1, AceUBR1, deltaSLF1, his3delta200, leu2-3,112, ura3-52) is transformed by standard methods with the NotI digested plasmid DNA (Figure 7). Cells are plated on CSM agar lacking histidine and are incubated at 30 °C for
20 40 to 48 hours. Single colonies are selected and restreaked for single colonies on the same media. A culture from a single colony is grown in YPD and a genomic DNA is isolated for evaluation by PCR reaction to verify the construction of the strain.

PCR primers for genomic verification are designed so that one primer (Primer 5') at one end of the gene is 5 prime to Primer 1 (above) used to generate the

shuffled gene on the plasmid and on the same strand as Primer 1. Another primer (Primer 3') is designed which is 3 prime to Primer 4 (above) and on the same strand as Primer 4. PCR conditions are as those described for Primary PCR (above). Another primer, specific for the plasmid sequence, 5'-ACCCTGGCGCCCAATACG-3' (SEQ ID NO:23), is used in conjunction with Primer 3' to amplify DNA from the mutant strains, i.e., those that contain the shuffled genes. The product of this PCR reaction is typically 600 to 700 base pairs in length.

Wild type genomic DNA and Primer 5' and Primer 3' are used to amplify a 1 to 1.5 kb PCR product. This primer pair should not yield a PCR product from the mutant genomic DNA using these PCR conditions because the product would be too big to amplify (>7 kb).

Example 5: Assay for Reversion Frequency in Yeast Strains Engineered with Copper Repressible Genes

The following assay was performed to assess the frequency with which a culture maintained under non-selective conditions will revert to a phenotype of non-sensitivity to exogenously added metal ions. Cultures of strains produced by two different methods in different strain backgrounds were grown in the absence of selection, then assayed to determine what percentage of the cells in the culture were no longer sensitive to the addition of copper ions.

Two independent isolates of ZMY71(ZM71 #1 and ZM71 #2) were used in this assay. ZM71 is derived from ZMY60, and its construction is described in Moqtaderi, Z. et al., *Nature* 383:188-191 (1996). The SUA7 gene was operably linked to an ANB1 promoter by a single cross-over strategy in a strain in which ROX1 and UBR1 are activated by the addition of copper to the culture medium. The recombinant strains are

maintained by selection on media lacking uracil (-URA). It is known that in the absence of selection, spontaneous recombination results in a strain in which the URA3 gene is lost (reverting to a ura3 phenotype) and regulation of the SUA7 gene by the ANB1 promoter is lost, while wild-type regulation of SUA7 is restored.

5 Two independent yeast strain isolates (19SG1 and 19SG2) were also used. In these yeast strains the SUA7 gene in strain CUY106 was operably linked to an ANB1 promoter by the double cross-over strategy as detailed in Example 4. The recombination results in a strain that can be selected for on media lacking histidine (-HIS). Because of the method used to engineer this strain results in an insertion which does not contain any
10 tandem repeats of sequence, it should be less likely that in the absence of selection on -HIS media the strain would revert to a his3 phenotype or regain wild-type regulation of the SUA7 gene.

 All strains were streaked from glycerol stocks to the appropriate selective media (uracil free media for ZM71 and histidine free media for the 19SG strains) and
15 were grown for 72 hours at 30°C. Single colonies were picked and inoculated into 2 ml of selective media and were cultured overnight in a rollerdrum at 30°C. The yeast cultures were microcentrifuged for approximately 5 seconds and the pellets were resuspended in two ml of YPD media (non-selective). The cultures were then grown 24 hours at 30°C in a rollerdrum.

20 Dilutions of each culture were plated to YPD and CSM plus 1 mM cupric sulfate plates. Plates were incubated for 72 hours at 30°C and the colonies were counted.

YPD plate colony numbers reflect the total cells in the culture, while colonies on the CSM plus 1 mM cupric sulfate plates indicate revertants, i.e., cells which have become

insensitive to the copper ion stimulus. Revertants are expressed in the table below as a percentage of total cells observed.

	<u>STRAIN</u>	<u>REVERTANTS</u>
5	ZM71 #1	0.012%
	ZM71 #2	0.22%
	19SG1	0.00024%
	19SG2	0.00042%

10 Not all reversions are due to genetic changes at the SUA7 locus. It is also possible that the copper stimulation of UBR1 or ROX1 gene expression can be ablated. However, since the control of these genes is identical in all the strains, any ablation of copper stimulation of these genes appear as background which all strains will share. The change in the reversion frequency at 24 hours in the 19SG strains engineered according
15 the methods of Example 4 demonstrates the improvements that can be achieved by altering gene expression in yeast according the methods disclosed herein.

Example 6: Construction of Yeast Strains Containing CYC8-LexA Repressor
Under Copper Control

20 The following procedures are performed to produce a yeast strain that expresses a heterologous repressor under copper control. Such strains avoid the potential problems of ROX1-based repressor strains, which include the pleiotropic effects and toxicities of ROX1 and metal ions. In a LexA-based repressor system, the addition of metal ions represses only recombinant genes whose promoters have been engineered to
25 contain the bacterial-derived recognition sequence for LexA (LexA operator). A CYC8-LexA fusion has been shown to repress the transcription of a yeast gene when the

LexA operator sequence is placed adjacent to the promoter of the yeast gene (Keleher et al., *Cell* 68:709, 1992).

This system comprises two components: (i) a yeast strain which, in the presence of copper, expresses a CYC8-LexA fusion protein or a fusion protein between
5 LexA and a fragment of ROX1 that lacks DNA-binding activity and (ii) a DNA fragment which renders any desired target gene repressible by LexA when introduced upstream of the start of the open reading frame. Notably, this can be achieved even if only a limited amount of sequence information is available.

A repressor fusion protein is constructed so that the DNA-binding domain
10 of LexA (amino acids 1-87) is fused to the N-terminus of the entire CYC8 protein (amino acids 1-966) as well as 23 amino acids derived from the 5' untranslated region of CYC8. This hybrid protein is expressed from a conditionally "inert" locus, such as TRP1. Alternatively, a fusion protein is constructed so that the DNA-binding domain of LexA is fused to a ROX1 protein which has been mutated so that it no longer binds to ROX1
15 recognition sequences, such as those present in the yeast ANB1 promoter.

In order to render a particular gene repressible by the hybrid repressor proteins described above, a generic repressible promoter cassette is designed that can be inserted upstream of any gene. The promoter cassette consists of one to several copies of the LexA operator placed upstream of a UAS-containing yeast promoter, such as, e.g.,
20 CYC1, adjacent to a gene such as HIS3 which can be used for positive selection. The insertion cassette can be produced by a single- or double-round PCR strategy by analogy to the method shown in Figures 2A-C. In this case, the point of insertion is upstream of the promoter region and not at the translational start site (Figure 3).

The repressible promoter is modified so that it will integrate upstream of a given yeast target gene as follows. Four PCR primers are designed based on limited sequence data flanking the 5' end of the gene of interest as shown in Figures 2A-C.

5 (i) Primer A is located 100-200 base pairs upstream of the beginning of the open reading frame of the gene, oriented toward the gene.

(ii) Primer B is located 100-200 within the 5' end of the open reading frame, oriented towards the 5' start of the gene.

(iii) Primers C and D contain both sequences specific to each gene as well
10 as sequences homologous to the generic repressible promoter cassette. Primer C contains at its 5'-most end sequences corresponding to the 3' end of the promoter cassette, in this case the 3' end of the CYC1 promoter. The 3' half of primer C contains sequences corresponding to the 5' end of the open reading frame of interest. The 3' end of Primer D consists of the sequence complementary to the sequence just upstream of the gene of
15 interest. The 5' half of Primer D consists of sequences complementary to the left-most end of the promoter cassette, in this case the 3' end of the HIS3 gene.

Typical sequences for primers C and D are:

Primer C: 5

ACAAATACACACACTAAATTAATAATGNNNNNNN-3' (SEQ ID NO:19)

20 Primer D: 5'-end of HIS3- NNNNNNNNNNNNNNNNNNNNNNNN-3'

Two sets of PCR reactions are performed. In the first set, a fragment of DNA containing the gene of interest and 5' flanking region is used as a template and amplification is performed using Primers A and D or Primers B and C. The resulting fragments are then included in a second round of PCR containing the promoter cassette,
25 both initial PCR products, and Primers A and B. This results in a larger fragment

containing the promoter cassette flanked by pieces of DNA that will target the DNA just upstream of the gene of interest (Figure 3).

Following transformation into yeast and selection for integration, stable integrants produced by sequence-specific recombination into the site of interest can be positively identified by PCR or Southern blot analyses. This strategy produces a conditional locus that is repressible by the LexA fusion proteins described above.

Example 7: Demonstration of Cidal and Static Effects of Copper Ion-induced Repression of Gene Expression

Three different yeast cell strains were constructed based on the CUY106 strain. The yeast CDC15, SUA7, and ERG11 genes were rendered repressible by copper-ion addition to the growth medium as described above.

The viability of each strain was evaluated at several time points over an 8 hour period after the addition of 1 mM copper sulfate to the growth medium by diluting the cells and plating them on YPD medium, without copper sulfate. Yeast colonies were counted after 48 hours of incubation at 30 °C in order to determine the colony forming units (CFU) per ml of original copper sulfate-containing culture medium at the time the yeast cells were harvested and diluted. The CFU/ml value was divided by the measured absorbance of the original copper sulfate-containing growth medium at 600 nm (A_{600}) at the time the aliquots were taken for dilution and plating onto YPD medium, yielding a viability index of CFU/A_{600} . The limit of detection for CFU/A_{600} is approximately 1×10^5 . Figure 11 shows the results of the assay, demonstrating that repression of the expression of some genes, such as SUA7 and ERG11, is cidal, i.e., kills, the yeast cells, while repression of expression of other genes, such as the CDC15 gene, only has a static effect on the yeast cells, arresting their growth but not killing them.

Example 8: The Copper Regulated Strains Are Complemented

Wildtype and mutant forms of BOS1 were created using PCR. The wildtype Bosl gene was amplified from *Saccharomyces* genomic DNA in a one-step reaction using the following oligos: ScBos1-1 (5'-
5 GCGGCTCGAGGGGTTTTCTCTCAACATTG-3') [SEQ ID NO: 25] and ScBos1-2 (5'-
GATCGCGGCCGCGTAAGGCTTATTGCTGCG 3') [SEQ ID NO: 26]. The region cloned included the wildtype BOS1 promoter and contained XhoI and NotI restriction sites at the 5' and 3' end respectively to facilitate cloning. The mutant allele, bosl-200, is missing codons 9-130 and was created by PCR in two steps using four oligos. In the first
10 round of amplification, the 5' region of the gene was amplified using oligos ScBos1-1 and ScBos1deIr (5'-CCACCAACGTTCTCACAGCATGGTTGTAAAGAGC-3') [SEQ ID NO: 27] and the 3' portion of the gene was amplified using oligos ScBosldelf (5'-AGGAACGTTGGTGGTGCG-3') [SEQ ID NO: 28] and ScBos1-2. In the second round, the two products of the first round of amplification were used as template and were
15 amplified using primers ScBos11 and ScBos1-2. The genes were cloned into the yeast expression plasmid pRS315 (Sikorski, R.S. and Hieter, P., *Genetics* 122 19-27, 1989) containing the LEU2 selectable marker and a CEN sequence that functions to limit the plasmid copy number to one or two copies per cell. The BOS1 copper regulated strain, Y360, was transformed with pRS315, pRS315 expressing wildtype BOS1p, and pRS315
20 expressing the bosl-200 mutant protein.

The three strains, Y360, Y360 expressing wildtype BOS1 and Y360 expressing the bosl-200 mutant protein were streaked for single colonies on CSM plates and CSM plates containing 1mM CUS04. The plates were incubated at 30°C for 3 days

to allow colony formation. The results of the complementation of growth experiment are shown in Figures 14a-c.

In the absence of copper, levels of BOS1p were sufficient in the Y360 copper regulated strain and cells grew normally. In the presence of copper, BOS1 protein levels dropped and the cells were unable to survive. Thus, the Y360 strain containing the pRS315 vector alone died in the presence of copper. The Y360 strain expressing wildtype BOS1 was capable of growth on copper. The Y360 expressing the mutant bosl-200 protein was also capable of growth on copper. These result suggest that the bosl-200 mutation is not deleterious under these conditions. These generally applicable analyses can be used to test a region or regions of a given target protein for its ability to complement. The same methods are used to test a non-homologous cDNA for its ability to complement a Cu-regulated mtuant target gene by inserting the nonhomologous gene into a plasmid and carrying out the transformation of a given strain of the invention according to the methods described herein.

Example 9: Phenocopy control for compound treated cells

Two different yeast cell strains were constructed based on the CUY106 strain. The yeast TAF145 (Y002) and RPC34 (Y038) genes were rendered repressible by copper-ion addition to the growth medium as described above. The parent CUY106 strain and the TAF145 strain were used as controls. RPC34 is an essential gene encoding the C34 subunit of RNA polymerase III. RNA polymerase III is a multisubunit enzyme responsible for the synthesis of tRNAs, 5sRNAs and some other small RNAs. Rpc34 temperature sensitive and cold-sensitive mutants have been shown to have defects in tRNA synthesis. (Stettler, S., *et al.*, *J. Biol. Chem.* 267 21390-21395, 1992; and Brun, I., *et al.* *EMBO J.* 16 5730-5741 1997.) TAF145 is a component of general transcription factor IID (TFIID) and is required for synthesis of some mRNAs but is not required for tRNA synthesis. All of the strains used in this experiments were assayed for the production of tRNA upon addition of copper to the media by monitoring the generation of tRNA precursor.

Strains and Procedures:

Yeast Strains

CUY106 (Wild Type): Ace-ROX1, Ace-UBR1, Dslf1, his3D200, leu2-3,112, ura3-52

- 5 Y038 (RPC34): Ace-ROX1, Ace-UBR1, Dslf1, his3D200, leu2-3,112, ura3-52, ANB1p-Ub- Rpc34::HIS3

Y002 (TAF1145): Ace-ROX1, Ace-UBR1, Dslf1, his3D200, leu2-3,112, ura3-52, ANB1p-Ub-Taf1145::HIS3

10

Growing Yeast Strains

1. Grow yeast strains overnight in Yeast Extract Peptone and Dextrose Medium "YPD" at 30°C.
2. Dilute cultures in the morning to $OD_{600}=0.1$ and let them grow for 1hr at 30°C.
- 15 3. Measure OD_{600} (equal to about 0.2) and save aliquots of each culture for "0hr" point. Spin each aliquot for 5min at 5,000rpm at 4°C, discard supernatant. Resuspend pellet in 1ml ice-cold water. Transfer to a clean 1.5ml microcentrifuge tube. Microcentrifuge 10sec at 4°C, and remove supernatant. Freeze the pellet by placing tube on dry ice, and then transfer it to -80°C for storage.
- 20 4. Split cultures in two halves and induce one half with 0.75mM CuSO₄, continue growing both halves at 30°C.
5. Collect culture aliquots at 30min, 1hr, 2hrs, and 4hrs (or 3.5hrs) after induction with CuSO₄ (or a compound). Measure OD_{600} at each point (for growth curves). Wash cells in 1ml ice-cold water and freeze the pellet by placing tube on dry ice. Store at -
25 70°C.

Preparation of Yeast RNA with Hot Acidic Phenol

1. Thaw pellet on ice. Resuspend pellet in 400µl TES solution (10mM tris-Cl, pH 7.5; 10mM EDTA; 0.5% SDS). Add 400µl acid phenol (Gibco BRL) and vortex vigorously 10min. Incubate 30 to 60 min at 65°C with occasional, brief vortexing.
- 5 2. Place on ice for 5 min. Microcentrifuge 5 min at top speed, 4°C.
3. Transfer aqueous (top) phase to a clean 1.5-ml microcentrifuge tube, add 400µl acid phenol, and vortex vigorously. Repeat step 2.
4. Transfer aqueous phase to a new tube and add 400µl of chloroform. Vortex vigorously and microcentrifuge 5min at top speed, 4°C.
- 10 5. Transfer aqueous phase to a new tube; add 40µl of 3M sodium acetate, pH 5.3, and 1ml of ice-cold 100% ethanol and precipitate. Microcentrifuge 5min at top speed, 4°C. Wash RNA pellet by vortexing briefly in 1ml ice-cold 70% ethanol. Microcentrifuge as before to pellet RNA.
6. Resuspend pellet in 50µl H₂O (heat if needed for resuspending at 65°C).
- 15 Determine the concentration spectrophotometrically by measuring OD₂₆₀ and OD₂₈₀. Store at -20°C.

$$(\text{RNA concentration} = \text{OD}_{260} / 0.0025 \mu\text{g/ml})$$

7. Equalize RNA concentration in all samples to 2µg/µl:
8. Run 2µl of each RNA sample on 0.7% agarose gel to visualize equal RNA concentrations.
- 20

S1 Nuclease Assay

1. tRNA Oligonucleotide Probe Labeling

Mix 2µl H₂O with 1µl of unlabeled 5µM oligo tRNA^w

(5'-GGAATTTCCAAGATTTAATTGGAGTCGAAAGCTCGCCTTA-3' [SEQ ID NO:29]. This sequence is complementary to the 5' intron-exon junction of short-lived precursor tRNA (Cormack and Struhl, Cell 69, 685-696,1992).and contains six nucleotides at the 3' end which are noncomplementary to RNA in order to carry out the S1 nuclease assay.

Incubate at 70°C for 5min, cool on ice, spin to collect sample on the bottom.

Add to the tube:

2.5µl gamma-³²P-ATP (>5000 Ci/mmol, 10 µCi/µl)

10µl 10x protein Kinase "PNK" buffer (NEB)

10 1µl PNK (NEB)

Incubate at 37°C for 45min.

Incubate at 70°C for 15min. Store on ice or at -200°C long term.

2. Hybridization

Use 40µg of RNA per reaction

15 RNA

10µl 5x hybridization buffer (1.5 M NaCl; 5mM EDTA; 190 mM HEPES, pH 7.0)

1µl 5% Triton X-100

1µl labeled probe

20 H₂O to 50µl total volume

Incubate 15min at 75°C, and then 4 hours or overnight at 55°C

3. S1 Nuclease Digestion

After hybridization is complete, to 50µl hybridization mix add:

50µl 10x S1 buffer (3M NaCl; 20mM ZnOAc; 600mM NaOAc, pH 4.5)

2µl 5% Triton X-100

400µl H₂O

150units S1 nuclease (0.375µl of 400U/µl S1 from Boehringer Mannheim)

Incubate at 37°C for 30min

- 5 Add 5µl of 10mg/ml in H₂O carrier bakers yeast tRNA and 5µl of 0.5M EDTA, pH8.0, and precipitate with 1ml of 100% ethanol for 30min at -70°C. Spin 20min in a microfuge; remove supernatant and dry pellet on benchtop for 10-15min. Add 12µl sequencing loading buffer, heat 5min at 100°C and put on ice for 5min. Load 4µl on 10% TBE-Urea gel (BIORAD). Run at 200V for 20 minutes.
- 10 Dry gel in a vacuum drier for 2 hours at 80°C. Reveal results by autoradiography.

- Figures 15 demonstrates that, upon addition of CuSO₄, RNA synthesis drops in the Cu-regulatable RPC34 strain (YO38). tRNA synthesis was not decreased upon addition of Cu in the control CUY106 strain or the TAF145 regulatable strain (Y002). This result is consistent with published reports on the role of RPC34 in tRNA synthesis. (Brun et al., *EMBO J*, **16**:5730, 1997) and demonstrates that the regulatable strains of the invention can be utilized to provide information regarding the phenotypes expected when antifungal compounds are isolated.
- 15

20 **Example 10: Drug Sensitivity Profiling**

- In *S. cerevisiae*, the essential gene *ALG7* encodes the enzyme Dolichol-P-dependent N-acetylglucosamine-1-P transferase (GPT). The GPT enzyme initiates the first step in the assembly of dolichol-linked oligosaccharide N-glycosylation. Kukuruzinska *et al.*, *Biochim Biophys Acta* **1247**(1) 51-59 (1995)
- 25 demonstrated that downregulation of *ALG7* expression results in diminished N-glycosylation and hypersensitivity to tunicamycin. More recently, Giaever *et al.*

described the increased sensitivity of an *ALG7/alg7* heterozygous diploid *S. cerevisiae* strain to tunicamycin. The analysis described below showed that when the gene which encodes the dolichol-P-dependent N-acetylglucosamine-1-P transferase (GPT) was down regulated by the presence of copper, sensitivity to tunicamycin was increased. The studies demonstrate that lowering the dosage of an essential gene results in a population sensitized to compounds that acts on the product of that gene.

In a single 96 well assay plate, a serial dilution of copper sulfate was created from right to left. On the same plate a serial dilution of compound was created along the opposite axis to give the final compound and copper concentrations.

Each well was inoculated with 10^3 cells/mL. Yeast cell growth was scored by eye after 48 hours incubation at 37° C.

Plates were setup as described above and inoculated with wild-type, copper regulated *ALG7* and copper regulated *CHS3* strains. *CHS3* encodes chitin synthase and provides a second control. Following 48 hours incubation at 37° C, plates were examined to assess growth (Figure 16). The studies showed that only the *ALG7* copper regulated cells demonstrated increased sensitivity to tunicamycin as the copper concentration was increased (Figure 16, panel a). No increase in sensitivity to tunicamycin was seen when non-target specific genes were down regulated, i.e. *CHS3* (Figure 16, panel b) or wild type (Figure 16, panel c) controls. These data demonstrate that copper acts synergistically with the antifungal agent tunicamycin and that the increased tunicamycin sensitivity is due to a reduction of the *ALG7*-encoded target.

Other strains engineered to regulate previously identified as drug targets were also examined. The copper regulated gene, the function of the target protein, and the drug tested are provided in the table below. The *ALG7* and tunicamycin experiment was repeated as a positive control.

GATE strain	Function of target	Compound
Cu <i>ERG11</i>	ergosterol synthesis	Azole
Cu <i>TUB1</i>	polymerization of tubulin	Benomyl
Cu <i>CMD1</i>	Ca ²⁺ regulation via calmodulin	Fluphenazine
Cu <i>ALG7</i>	N-Glycosylation	Tunicamycin

The results of this analysis are shown in Figure 17, panels a-d. In each case the strain demonstrated greater sensitivity to the compound as the copper concentration was increased. These experiments show that the strains of the invention can be used to identify target genes for antifungal compounds and provide tools for the analysis of antifungal agents and their mechanisms of action. Rapid reduction of antifungal target gene products lead to increased sensitivity to that agent and provide a phenotype by which to analyze the possible mechanisms by which the antifungal agents acts.

Claims:

1. A method for generating a yeast strain comprising a regulated target gene comprising:

(a) generating a yeast cell comprising

(i) a first gene encoding a transcriptional repressor protein whose expression is under the control of a metal ion-responsive element, wherein expression of said first gene encoding said repressor protein is stimulated by the addition of a metal ion to growth medium of said yeast cell;

(ii) a second gene encoding a subject protein, wherein expression of said second gene encoding said subject protein is controlled by a transcriptional control sequence whose activity is inhibited by said repressor protein; and

(iii) a third gene encoding a biomineralization protein, wherein said third gene is inactivated and wherein inactivation of said third gene enhances transcriptional response of said metal ion-responsive element to metal ions in said growth medium of said yeast cell;

(b) culturing the yeast cell in a growth medium comprising metal ions, wherein said metal ions are present in sufficient concentration to activate said metal ion-responsive element to a level which will result in said predetermined level of repression of expression of said subject gene;

(c) assessing whether the rapid depletion of the second gene from the yeast cell leads to inhibition of cell growth or cell death.

2. The method of claim 1, wherein said inhibition identifies the target gene as an essential target gene.

3. The method of claim 1, wherein said transcriptional repressor protein is the protein encoded by the ROX1 gene.

4. The method of claim 1, wherein said transcriptional control sequence is ANB1 promoter.

- 1 5. The method of claim 1, wherein said biomineralization gene is SLF1.
- 1 6. The method of claim 1, wherein the yeast cell further comprises a fourth
2 gene encoding a protein that targets ubiquitin-containing polypeptides for degradation.
- 1 7. The method of claim 6, wherein said fourth gene is placed under the
2 control of a metal ion-responsive element.
- 1 8. The method of claim 6, wherein said fourth gene is UBR1.
- 1 9. The method of claim 1, wherein said transcriptional repressor protein is
2 CYC8-LexA.
- 1 10. The method of claim 1, wherein said gene encoding said transcriptional
2 repressor protein is ROX1-LexA.
- 1 11. A method of screening a candidate antifungal compound for interaction
2 with an essential target gene comprising:
3 (a) generating a regulated yeast strain comprising a regulated essential
4 target gene as defined in claim 1;
5 (b) establishing a concentration of metal ion at which the growth or
6 viability of the regulated yeast strain ceases;
7 (c) generating a serial dilution of metal ion in yeast growth media;
8 (d) culturing the regulated yeast strain in the serially diluted growth media,
9 wherein the serial dilution leads to a dose-dependent modulation of expression of the
10 regulated essential target yeast gene product;
11 (e) screening the serially diluted cultures for altered sensitivity of the
12 strain to the candidate antifungal compound;
13 (f) determining the metal ion concentration present in a culture
14 demonstrating altered sensitivity to the candidate antifungal compound;
15 (g) comparing the metal ion concentration of step (b) with the metal ion
16 concentration of the culture determined in step (f), and identifying a candidate antifungal

7 compound for which a lower concentration of metal ion is required to eliminate growth
8 or viability in step (f) as compared to step (b).

1 12. The method of claim 11, wherein a plurality of candidate antifungal
2 compounds are screened.

1 13. The method of claim 12, wherein the screen of the plurality of candidates
2 is selected from the group consisting of a screening together in a single assay and a
3 screening individually using multiple simultaneous individual detecting steps.

1 14. The method of claim 11, wherein said transcriptional repressor protein is
2 the protein encoded by the ROX1 gene.

1 15. The method of claim 11, wherein said transcriptional control sequence is
2 ANB1 promoter.

1 16. The method of claim 11, wherein said biomineralization gene is SLF1.

1 17. The method of claim 11, wherein the yeast cell further comprises a fourth
2 gene encoding a protein that targets ubiquitin-containing polypeptides for degradation.

1 18. The method of claim 15, wherein said fourth gene is placed under the
2 control of a metal ion-responsive element.

1 19. The method of claim 15, wherein said fourth gene is UBR1.

1 20. The method of claim 11, wherein said transcriptional repressor protein is
2 CYC8-LexA.

1 21. The method of claim 11, wherein said gene encoding said transcriptional
2 repressor protein is ROX1-LexA.

1 22. A method of rapidly cloning a DNA complementary to an essential target
2 gene comprising:

 (a) generating a regulated yeast strain comprising a regulated essential
target gene as defined in claim 1;

 (b) establishing a concentration of metal ion at which the growth or
6 viability of the regulated yeast strain ceases;

 (c) transforming the regulated yeast strain with a DNA to be tested for
complementation;

 (d) culturing the transformed regulated yeast strain in growth media
containing a concentration of metal ion as established in step (b);

11 (e) determining the ability of the DNA to complement the regulated
essential target gene, wherein growth or viability of the regulated yeast strain establishes
complementation; and

 (f) cloning the complementary DNA.

1 23. The method of claim 22, wherein the DNA is selected from the group
2 consisting of genes from another organism, mutant DNA and DNA fragments.

3
4 24. The method of claim 23, wherein the organism is selected from the group
5 consisting of human, mouse, mammal, drosophila and mycete.

1 25. The method of claim 22, wherein the DNA is generated from the group
2 consisting of genomic and cDNA libraries.

1 26. The method of claim 22, wherein said transcriptional repressor protein is
2 the protein encoded by the ROX1 gene.

1 27. The method of claim 22, wherein said transcriptional control sequence is
2 ANB1 promoter.

1 28. The method of claim 22, wherein said biomineralization gene is SLF1.

1 29. The method of claim 22, wherein the yeast cell further comprises a fourth
2 gene encoding a protein that targets ubiquitin-containing polypeptides for degradation.

1 30. The method of claim 29, wherein said fourth gene is placed under the
2 control of a metal ion-responsive element.

1 31. The method of claim 29, wherein said fourth gene is UBR1.

1 32. The method of claim 22, wherein said transcriptional repressor protein is
2 CYC8-LexA.

1 33. The method of claim 22, wherein said gene encoding said transcriptional
2 repressor protein is ROX1-LexA.

1 34. A method of determining an antifungal effect of an antifungal compound
2 comprising:

3 (a) generating a regulated yeast strain comprising a regulated essential
4 target gene as defined in claim 1;

5 (b) establishing a concentration of metal ion at which the growth or
6 viability of the regulated yeast strain ceases;

7 (c) culturing the regulated yeast strain in growth media containing the
8 concentration of metal ion as established in step (b);

9 (d) determining the phenotype associated with the culture of step (c) that
10 is depleted of the essential target gene;

11 (e) culturing a yeast strain in growth media with a candidate antifungal
12 compound;

13 (f) determining the phenotype associated with the culture of step (e) that
14 is treated with the candidate antifungal compound;

15 (g) comparing the phenotypes determined in steps (d) and (f) to determine
16 the antifungal effect of the antifungal compound.

1 35. The method of claim 34, wherein determining the phenotypes comprises

- 2 (i) incubating the cultures with radio-labeled macromolecular
3 building-blocks;
4 (ii) establishing a level of incorporation of the radio-labeled
5 macromolecular building blocks for each culture; and
6 (iii) analyzing the macromolecular products generated in each
7 culture.

1 36. The method of claim 34, wherein said transcriptional repressor protein is
2 the protein encoded by the ROX1 gene.

37. The method of claim 34, wherein said transcriptional control sequence is
ANB1 promoter.

1 38. The method of claim 34, wherein said biomineralization gene is SLF1.

1 39. The method of claim 34, wherein the yeast cell further comprises a fourth
2 gene encoding a protein that targets ubiquitin-containing polypeptides for
3 degradation.

1 40. The method of claim 39, wherein said fourth gene is placed under the
2 control of a metal ion-responsive element.

1 41. The method of claim 39, wherein said fourth gene is UBR1.

1 42. The method of claim 34, wherein said transcriptional repressor protein is
2 CYC8-LexA.

43. The method of claim 34, wherein said gene encoding said transcriptional
repressor protein is ROX1-LexA.

1/25

FIG. 1

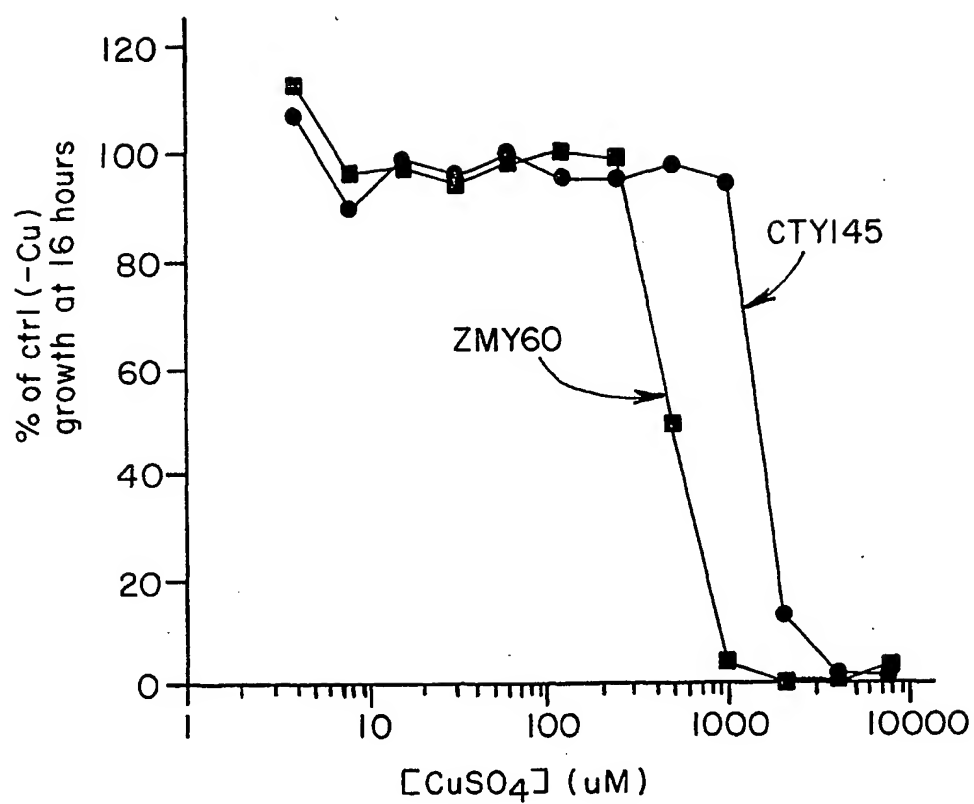
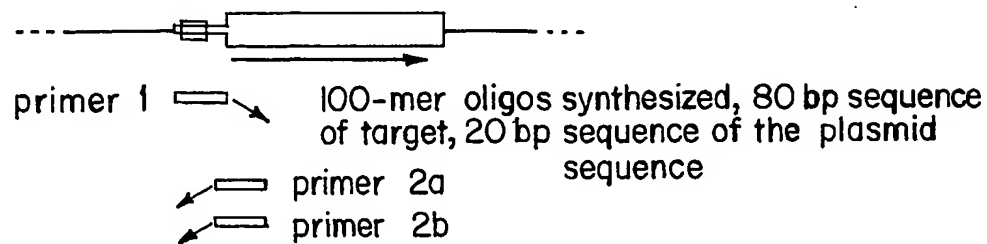
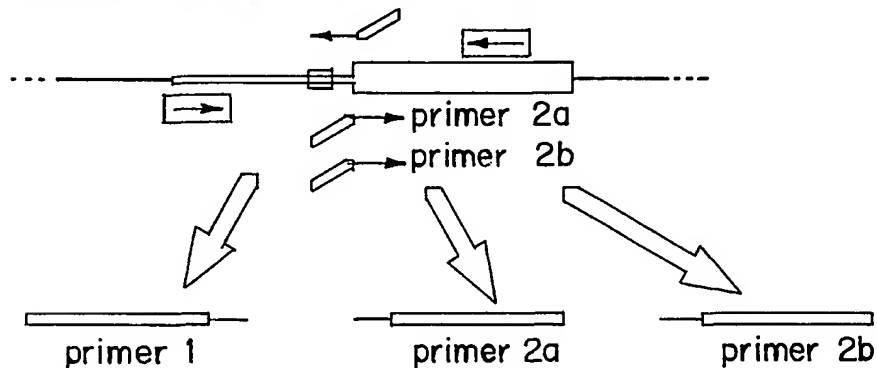


FIG. 2A

A. SINGLE ROUND PCR STRATEGY (OLIGOS SYNTHESIZED):



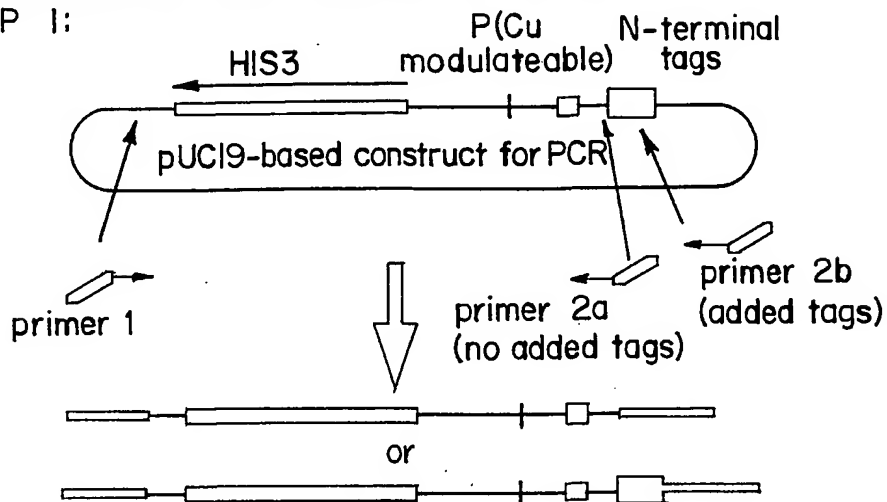
B. DOUBLE ROUND PCR STRATEGY (OLIGOS PRODUCED BY PCR)



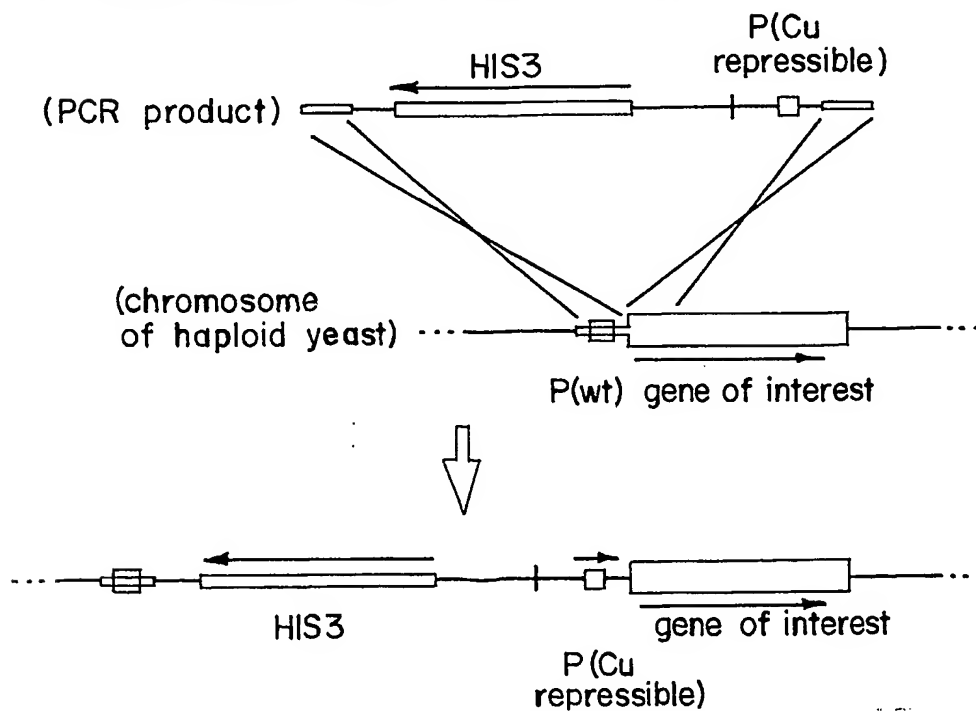
3/25

FIG. 2B

II: TRANSFORMING DNA PRODUCED BY PCR WITH OLIGOS FROM STEP I:

**FIG. 2C**

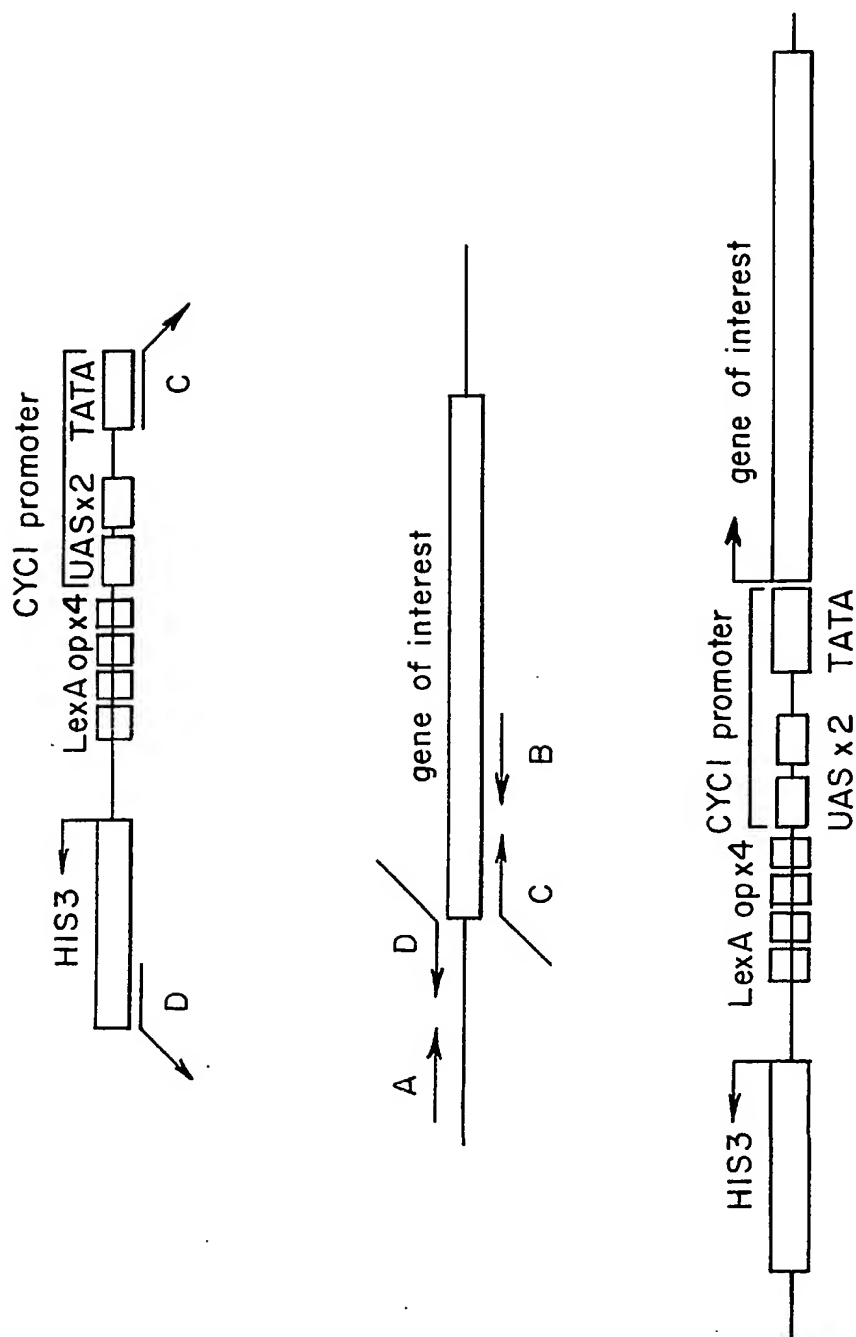
III: TRANSFORMATION, RECOMBINATION



SUBSTITUTE SHEET (RULE 26)

4/25

FIG. 3



GAATTAAATTCGAGCTCGGTACGGTGATCTTCGTCGGCCACAAATCCCCTGGATATCATTTGGCC
TGTCGAGGTATCGGCCGCGTGAACCTACCGGAATTACTATGCAAAACAAATTGAAATCTGGTAG
GAAAACCTTGTTCTAGAACCTTGGCGATTGCTGACAAAGAAAGAGGCCCTATTGTTGCTGCCCTC
TTTTGTTGTTCTTCTCCTGATATGCTTGGCGGTGTTCTTTGTTGTTCTTTGTTGTTCTTTAC
TATTATAGTGTCTTTTGCTATTATATTTCTTCTGTTTTCACCTTTGCGTAATGTAACGGTCTTAAA
CAAAAGTTTTTTTTTTTCGCTCTTGCAATTTCCCTTTTCTGCTCTATCTTATTTGCTAAATTGTAGT
TTTCAGAAAGTTTTTACCTTAAATATAGCACTATTTCCAGTTTTTAAATGTTTCTTCTCATTTGCTTTCT
TTTATAAATTTTCGCATATAAATATACATTTACGGTGTCTTAACTCTCCCTCTTCACCCCTCATTTA
TTCCAGAAAAATACATAACTCTTCCACACAAAAGAACGCAGTTAGACAAATCAACAAATGACTAGTA
GTTTTTCTTGAACCAAAGAAAGGTCACAGAGGCAATAGACTCTTCAATCTCATTTGATCTCTTGC
TTTGGCTTCTGCAGTGGACGAGAACTTGGCCCTTTTGGCCCTTAAATAGAGTCGTGAATGTTGCTTCTCAATTTGGTTGTTTTT
TCTCTTGATTTTGAGCATCCAATTGCTTAAATAGAGTCGTGAATGTTGCTTCTTACGTTCTTGTGTTGTTCAAGT
CAGCTTGATCTTGATGATCTCCTTGTTCTTATCCTGTAACTTCTTACGTTCTTGTGTTGGTGGTA
TCGTTGACCTGTTGATCGATTTGCTTTCTAATTAAACCGAATTCAGTGTGCTGATTTTCTTCAA
TTGAAACGTTAAGAGTGTCCAATTTCTTGCTCTTAAACGGAGACATCTGGGCGCTTGAACCTTGTGTT
GTTGGAGGACATGGCAATGGCTGTGTTGTAGAAAAATATGCTATTACGTTGATAAAAGAGGAA
AGGTGAAATCAGTTCAAAAATGTGAATGAAACTGAACGAAAGAAATGACAGAAATGAGTGAAAAAT
GGAGATGGAGGGCAAAATGAAAAAAGGATGAACCTAAAAATAGAAAAATAGACTCCGTC
GTACTTTAATGCTATGTATAACGCAACCAAGCAATTTTCGAAACTCAATTTGGCTTATAAATGTT
CGAGATAAAATGCGAATTAACGTGTTCAACGTCGTGAGATCAGTTATTTTTTTTTCACGCCACAGT
GCGGTAAAGCAATTTTTCGGGTACCAACCACTAACACATGATAATGATATAGGCTTATTATG
TATGTTTGTGCTACTTTATATGACGGTTATTTACAAGTTAGAAATATCTATTAAACAATGCGAGT
AGCCACGCTTACGTTTAGTGAGTCAACAAATGGGTTCTGGGCGCCGATTGCCCTTTCTCAATGCCAC
CAAAGGGAATTCGACGAAAGTCACTCTCATCTTCAAATTCGTTCTTACGCCCTGGCTTTTCG
TTCCCCACCACTAGAAACACAGGCAGCTCGTTACATAATCCGTTCAAATCGTGCAATGCTAAATAGT
TTTTCCAACACAGTGATTTTTCTGACGTGGCATTAGCTAAGTGCTTGTAAATAAACGTCAGCCAC
CCATTTCTTGATTTAGTAAAAAATCTAACGGTTTATCAACGTAAAAATATGGGCAGAAAGTTTCG
AGGCCCCCACTGCTTGTCTTGGACACCAACAGGCGTCAAAAGGAGAGCAGTTTCTTCTCGACATCA

A_____A

6/25

FIG. 4B

A
AATGAAGTCAACCCAGGAAGTAAGCGCTTCTTAATAATGGCACCCGATATTGTGAGGTGAGTTA
TTTCATCCAGATATAACCCGAGAGGAAACTTCTTAGCGTCTGTTTCGTACCATAAAGCAGTTCA
TGAGGTATATTTTCGTTATTGAAGCCAGCTCGTGAATGCTTAATGCTGCTGAACCTGTTATTTGTCA
GTCGCCCTAGGTACGCAATCTCCACAGGCTGCAAGGTTTGTCTCAAGAGCAATGTTATTGTGCA
CCCCGTAAATTGGTCAACAAGTTTAATCTGTGCTTGTCCACCAGCTCTGTCTGTAACCTTCAGTTCA
TCGACTATCTGAAGAAATTTACTAGGAATAGTCCCATGGTACAGCAACCGAGAAATGGCAATTTCT
ACTCGGGTTCAGCAACGCTGCATAAACGCTGTTGGTCCGTAGACATATTCGAAAGATAGGATTAT
CATTCATAAGTTTCAGAGCAATGTCCTTATTCTGGAACTTGGATTATGGCTCTTTTGGTTTAAT
TTCCGCTGATTCTTGATCTCCTTTAGCTTCTCGACGTGGGCCCTTTTCTTGCATATGGATCTGA
ATTCAGTCTTTTGTGGAACGTTGAGCGGAAAGACGCATCGAATTCGAGCTCGTTAGCGA
TTGGCATTATCACATAATGAATTATACATTATATAAAGTAATGTGATTTCTTCGAAGAATATACT
AAAAATGAGCAGGCAAGATAAACGAAGCAAGACGGTATCGATATCAATGAATCCTAAATCC
TCTACACCTAAGATTCCAAGACCCAGAACGCAATTATTCTGTTCAGACAGCACTACCAAGGAT
CTTAATAGACGAATGGACCGCTCAAGGTGTGGAAATACCCCATAATTCAAAATTTCTAAATTA
TTGGTACGAAGTGGAGGGCTTACAACCGGAAGATAAGGCACACTGGGAAATCTAGCGGAGAG
GAGAACTAGAACATGAAGGAAGTATCTGAATACAAATACAAAGCCGTTAAGAAAGTCTAAGAA
GAAGCAACTACTTTTGAAGGAATCGAGCAACAGCAGCAACACAGAAAGAACAGCAGCAGC
AGAAACAGTCAACAACCGCAATTACAACAGCCCTTTAAACAACATATAGTTCTTATGAAAAGAGCA
CATTCCTTTTCAACCATCTTCCCTCGGTGTCAAGCTCGAACAGCTATCAGTTCCAATTGAACAATGA
TCTTAAGAGGTTGCCCTATTCTCTCTGTTAATACTTCTAACTATATGGTCTCCAGATCCTCTAGAG
TCGACCTGCAGGCATGCAAGCTTGGCGTAATCATGTCATAGCTGTTTCCGTGTGAAATTGTTA
TCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGTGCTTAAT
GAGTGAGCTAACTCACATTAATTGCGTTGCGTCACTGCCGCTTCCAGTCGGGAAACCTGTGCG
TGCCAGGGGGATCCACTAGTTCTAGAGTCGACCGGCATGCAAGCTTGGCGTAATCATGTGTCATA
GCTGTTTCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAA
AGTGTAAGCCTGGGGTGCCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCC

B

7/25

FIG. 4C

B

GCTTTCAGTCGGGAAACCTGTGTCGTCAGCTGCATTAATGAATCGGCCAAACGCGGGGAGAGG
CGTTTTCGCTATTGGGCGCTCTTCCGCTTCTCCGCTCACTGACTCGTCGCTCGGTCGTTCCGGC
TGCGGCGAGCGGTATCAGCTCACTCAAAGCGGTAATA CGGTTATCCACAGAAATCAGGGGATAAC
GCAGGAAAAGAAATGTGAGCAAAAGCCAGCAAAAGCCAGGAAACCGTAAAAGCCCGTTGCT
GGCGTTTTCATAGGCTCCGCCCCCTGACGAGCATCAAAAAATCGACGCTCAAGTCAGAGGT
GGCGAAACCCGACAGGACTATAAAGATACCCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCT
CCTGTTCCGACCCCTGCCGCTTACCGGATACCTGTCCGCTTCTCCCTTCGGGAAGCGTGCGCT
TTCTCATAGCTCAGCTGAGGTATCTCAGTTCGGTGTAGGTCGTTTCGCTCCAAGCTGGGCTGTG
TGACGAAACCCCGTTAGCCCGACCGCTGCCCTTATCCGGTAACTATCGTCTTGAGTCCAAC
CCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAAACAGGATTAGCAGAGCGAGGTA
TGTAGCGGTGTACAGAGTTCTTGAAGTGGTGGCTAACTACGGCTACACTAGAAGACAGTAT
TTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGC
AAACAAACCAACCGTGGTAGCGGTGTTTTCGTTGCAAGCAGCAGATTACGCGCAGAAAAAA
AGGATCTCAAGAAGATCCTTTGATCTTTCTACGGGCTGACGCTCAGTGAACGAAAACTCAC
GTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACTAGATCTTTTAAATTAAAAA
TGAAGTTTAAATCAATCTAAAGTATATAGAGTAAACTTGGTCTGACAGTTACCAATGCTTAAT
CAGTGAGGCACCTATCTCAGCGATCTGTCTATTTTCGTTTCATCCATAGTTGCCCTGACTCCCCGTCG
TGTAGATAACTACGATACGGGAGGCTTACCATCTGGCCCCAGTGTGCAATGATACCGCGAGAC
CCACGCTCACCGGCTCCAGATTATCAGCAATAAACCAGCCAGCCGGAAGGCCGAGCGCAGAAG
TGGTCCCTGCAACTTATCCGCTCCATCCAGTCTATTAAATGTTGCCGGAAGCTAGAGTAAGTA
GTTCCGCGAGTTAATAGTTTGGCAACGTTGTTGCCATTGCTACAGGCATCGTGTGTACGCTCG
TCGTTTGGTATGGCTTCATTACGCTCCGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCAT
GTTGTGCAAAAAGCGGTAGCTCCTTCGGTCCCTCCGATCGTTGTCAAGAAAGTTGGCCGCGAG
TGTTATCACTCATGGTTATGGCAGCACTGCATAAATCTCTTACTGTATGCCATCCGTAAGATGC
TTTTCTGTGACTGGTGAGTACTCAACCAAGTCACTCTGAGAAATAGTGTATGCGGCGACCGAGTTG
CTCTTGCCCGCGTCAATACGGGATAATACCGGCCACATAGCAGAACTTTAAAGTGTCTCATCA
TTGGAAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATG
TAACCCCACTCGTGCACCCCAACTGATCTTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGC

C

8/25

FIG. 4D

C

C

AAAAACAGGAAGCAAAATGCCGCAAAAAGGAATAAAGGCGCACACGGAATGTTGAATACTCA
TACTCTTCCCTTTTCAATATATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATA
TTTGAATGTATTTAGAAAAATAAACAAATAGGGTTCGCGCACATTTCCCGAAAAAGTGCCACC
TGACGCTAAAGAAACCAATTAATCATGACATTAACTATAAAAATAGGCGTATCACGAGGCCAG
CTTTCAATTCAATTCAATCATCATTTTCTTTTATTTCTTTTGTGATTTCCGTTTCTTTGAAATTTT
TTTGATTCGGTAATCTCCGACAGAAAGAAACGAAAGGAGGACACAGACTTAGATTGGTATA
TATACGCATATGTAGTGTGAAGAAAACATGAAATTGCCCCAGTATTTAAACCCAACTGCACAGAA
CAAAAACATGCAGGAAACGAGATAAATCATGTGAAAGCTACATATAAGGAACTGTGCTGCTACT
CATCCTAGTCTGTGCTGCCAAGCTATTTAATAATCATGCACGAAAAAGCAAACTTGTGTGC
TTCATTGGATGTTCTGTACCAACGAGGAATTACTGGAGTTAGTTGAAAGCATTAGGTCCCAAAATTT
GTTTACTAAAAACACATGTGGATATCTTGACTGATTTTCCATGGAGGACAGTTAAGCCGCTA
AAGGCATTAATCCGCCAAGTACAAATTTTACTCTTCGAAGACAGAAAATTTGCTGACATTTGGTAA
TACAGTCAAAATTGCAGTACTCTGCGGTGTATACAGAAATAGCAGAAATGGCAGACATTACGAATG
CACACGTGTGGTGGCCCAAGTATTTGTAGCGTTTGAAGCAGGCGGCAAGAAAGTAACAAAG
GAACCTAGAGGCCCTTTTGATGTTAGCAGAAATGTCTCATGCAAGGCTCCCTATCTACTGGAGAATA
TACTAAGGGTACTGTTGACATTCGGAAGAGCGACAAAGATTTTGTATCGGCTTTATTGCTCAAA
GAGACATGGGTGGAAGAGATGAAGGTTACGATTGGTTGATTAAGACACCCGGTGTGGTTTAGAT
GACAAGGAGACGCATTTGGTCAACAGTATAGAACCCGTGGATGATGTGGTCTCTACAGGATCTGA
CATTAATATTGTTGAAGAGGACTATTTGCAAAAGGAAGGATGCTAAGGTAGAGGTGAACGTT
ACAGAAAAGCAGGCTGGGAAGCATATTTGAGAAAGATCGGCCAGCAAACTAAAAAACTGTATTA
TAAGTAAATGCATGTATACTAACTCAAAATTAGAGCTTCAATTTAATATATCAGTTATTACC
CGCCCTTTCGTCTCGCGCTTTTCGGTGATGACGGTGAAAAACCTCTGACACATGCAGCTCCCGGAG
ACGGTCAACAGCTTGTCTGTAGCGGATGCGGGAGCAGACAAGCCGTAGGGCGGTACGCGGG
TGTTGGCGGTGTTCGGGCTGGCTTAACATAATCGGGCATCAGAGCAGATTGTACTGAGAGTGCACC
ATATGCGGTGTGAATACCGCACAGATCGGTAAAGGAGAAAATACCGCATCAGGCGCCATTGCGCA
TTCAGGCTGCGCAACTGTTGGGAAGGCGGATCGGTGCGGGCTCTTCGCTATTACGCCAGCTGGC
GAAAGGGGATGTGCTGCAAGCGGATTAAGTTGGGTAAACGCCAGGGTTTCCCAGTCAACGACGTT
GTAAAAACGACGGCCAGT

9/25

FIG. 5A

GAATTAATTCGAGCTCGGTACCAAGTTGCCACACCAAAAAGTCGAAAAAGGCTAAGAAACCAAAAGAAATAA
GGTACTAAGTACCCAGGCGCTACTAAGACCAACGAGATTGCCACGAAACTAGAGGAAACCAAAATGTAAAG
CATAGCTTAATCCGTTTTCACGATTCTATAATAATAAATAAGAAAAAGATATATCATATAAAACGTTATAA
AATTAATAACCGGTAAAGTGTAGAAAAGTGATCGGACGGTTTATTTTCTCTCTTCCCTCTTGGGATTGAATTT
AACTTGCAGATAGTGACCATAAAGGCAACTACCCAGTGGCAACACAGTTTGTGATAAACGCCCAGTACATCAAC
GAGCGAGTATAAAGACTTTGGTACATTTTAAAAAGGAAACATATATTTGTTTCAATTGCTAGACCCCTTTTA
GTCTCACCTCAATAAAAATCTGCTTTATTCCTCATTTGGGCTTTTATTTCTTTAATTTTGCATACCTATAGCG
TGAAACTGGGCATTTAAACAAAAGCAAACTATTTTAATAGTAGCATCCTGCTTTTCTTGGCCCTCCTTCTT
ATTGCGATACATTAATAAGTTTTTTACCACCTTTTCTCTCTCACTGAAGTCCCTAACTTTACAGGTCACACA
TGAAAGTTTACTGTATCCTATTAGTTGACTATTTTCTGAAACCAAGAAAGGTACCAAGGCAATAGACTCT
AATTACATAGAACATTCCAACTAGTAGTTTCTTGAACCAAGAAAGGTACCAAGGCAATAGACTCT
TCAATCTCATTGATTCTTTGCTTGCTTCTGAGCATCCAATTGCTTAATAGAGTCGTGAATGTTGCTTCTACG
CAATTGCTGTTTCTCTTGATTGAGCATCCTGTTGTTTCTTATCCTGTAACCTTCTTACGTTCTTGTG
GGTTTCAAGTCAGCTTGGATCTTGATGATCTCCTTGTCTTATCCTGTAACCTTCTTACGTTCTGATTTTTC
GTGGTATCGTTGACCTGTTGATCGATTGCTTTCTTAATAAACCGATTTCACTGTCGATTTTTCATCA
ATTGAACGTTAAGAGTGTCCAATTCTTGTCTCTAACGGAGACATCTGGGCGCTTGAACCTTGTGTTGTTG
GGAGGACATGGCAATGGCTGTGTTGTTAGAAAATATGCTATTACGTTGATAAAAGGAGGAAAGGTGAAAT
CAGTTCAAAAATGTGAATGAAACTGAACGAAAGAAATGACCAAGAAATGAGTGAATAATGGAGATGGAGGGC
AAAAAGAAAAAAGGATGAACCTAAAAATAGAAAAATAGACTCCGTCGTACTTTAATGCTATGTAT
AACGCAACCAAGCAATTTTCGAAACTCAATTTGGCTTATAAATGTTTCGAGATAAAATGCGAATTACGTGT
TCAACGTCGTCGAGATCAGTTATTTTTCACGCCACAGTCCGGGTAAGCAATTTTTCGCGTACCACCA
CCATTACACATGTATAATAGGCTTATATGTATGTTTGTGCTACTTTATATAGCAGGTTATTAC
AAGTTAGAATAATATCTAATAACATGCGAGTAGCCACGCTTACGTTTAGTGAGTCAACAAATGGGTTCTGG
GGCCGATTGCTTCTCAATGCCACCAAGGGAATTTTCGACGAAGAGTCACTCCTCATCTTCAAAATTC
GTTCTTACGCCCTGGCTTTCGTTCCCAACCACTAGAAACCAAGGAGCTCGTTACATAATCCGTTCAAAAT
CGTGCATGCTAATAGTTTTCCAACAGTGTATTTTCTGACGTGGCATTAGCTAAGTGGCTTGTAAATAAA
CGTCCAGCCACCCATTTCTTGTGATTTAGTAAAAAACTCTAACGGTTTATCAACGTAATAATATGGGCAGA
AGTTCGAGGGGCCCCACTGCTTGCTTGGACACCAAGGCGTCAAGGAGAGCAGTTTCTTCTCGACATCA

A

10/25

FIG. 5B

A
CAATGAAGTCAACCCAGGAAGTAAGCGCTTCTAATAATGGCACCCGATATTTGTAGGGTCAGTTATTTTC
ATCCAGATATAACCCGAGAGGAAACTTCTTAGCGTCTGTTTTCGTACCATTAAGGCAGTTTCATGAGGTATA
TTTTTCGTTATTGAAGCCAGCTCGTGAATGCTTAATGCTGTAACCTGGTGTCCATGTCCGCTAGGTACG
CAATCTCCACAGGCTGCAAGGTTTTGTCTCAAGAGCAATGTTATTGTGCACCCCGTAATTGGTCAACAA
GTTTAACTCTGTGCTGTCCACAGCTCTGTGTAACCTTCAGTTTCATCGACTATCTGAAGAAATTTACTA
GGAATAGTGCCATGGTACAGCAACCGAGAAATGGCAATTTCTACTCGGTTTCAGCAACGCTGCATAAACGC
TGTTGGTGCCGTAGACATATTCGAAGATAGGATTTATCATTCATAAGTTTCAGAGCAATGTCCTTATTTCTG
GAACTTGGATTATGGCTCTTTTGGTTTAAATTCGCCCTGATTTCTTGATCTCCTTTAGCTTCTCGACGTGG
GCCTTTCTTGCCATATGGATCTGAATTCCTAGTCTTTTTCGTGGAACGTTGAGCGGAAAGACGCAT
CGAATTCGAGCTCGTTAGCGATTGGCATTATCACATAATGAATATACATATATAAAGTAATGTGATTT
CTTCGAAGAAATATACTAAATAATGAGCAGGCAAGATAAACGAAGCAAGGACGGTATCGATAAGCTTGG
GAAATCAAAATGCCAAGAAAGCGGAAGTCCATATGTACCCATACGACGTTCCAGACTACGCTTCTT
TGGGTGTTCTAGCCCAAGCTTGATATCGAAATTCCTGCAGCCCGGGGATCCTAACATGTCCGTTGCTGA
TGATGATTTAGGATCTTTACAAGGTCACATTAGGAGAACACTGAGGTCTATTTCATAACCTCCCTATTTT
AGGTATACGAGAGGTCCTACTGAAAGGCTGACATGAGCAGAGCCCTTAAAGAGTTTCATTACAGATATC
TATACTTTGTCTATTCTAACAGCGGAGAGAACTTACCCTACTTTATTCAATGCTCATCCAAAACAAAAT
ATCTAACCCAGAGCTTACTGTTTTTCTGACAGTTTAGAAGATGCTGTGGATATTGATAAGATAACATCT
CAACAACTATTCCGTTTTTAAGATAGATGAATCCAGAAATAGGAGACGTCCTAATAACATACCGGAAGAA
ATTGFGGAGGAAATTCAAAATAGGGGAACCTTTGTATAGGTGTCTAGTGTGGTTGCCATGATACCTTG
TGTGCTTTGTATTCTATTGTTTAAATCCAAAAGATCATGTGAATCATCATGTTTGTACCGATATATGTACT
GAATTCGATATCAAGCTTGCGTAAATCATGGTCAATAGCTGTTTCCCTGTGTGAATTTGTTATCCGCTCACA
ATTCACACAAATACGAGCCGGAAGCATAAAGTAAAGCCCTGGGTGCCATATGAGTGAGCTAACTCA
CATTAATTGCGTTGCGCTCACTGCCCGTTTTCCAGTCGGGAAACCTGTCTGCCAGGGGGATCCACTAG
TTCTAGAGTCGACCGGCAATGCAAGCTTGGCGTAAATCATGGTCAATAGTGTGTTTCTGTGTGAATTTGTTAT
CCGCTCACAATTCACACAAATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGTGCCCTAATGAGTGA
GCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCTGTCAGCTGCA

B

11/25

FIG. 5C

B B

TTAATGAATCGGCCAACGCGCGGGAGAGCGGTTTGCGTATTGGCGCTCTTCCGCTTCCCTCGCTCACT
GACTCGTCGCTCGGTCGTTCCGCTGCGGCGAGCGGTATCAGCTCACTCAAAGCGCGTAATACGGTTAT
CCACAGAAATCAGGGGATAACGACAGGAAACATGTGAGCAAAAGCCAGCAAAAGCCAGGAACCGTAA
AAGGCCGCTGCTGGCGTTTTCATAGGCTCCGCCCTGACGAGCATCACAAAATCGACGCTCA
AGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGC
GCTCTCCTGTTCCGACCCGTCCGCTTACCGGATACCTGTCCGCCCTTCTCCCTTCGGGAAGCGTGGCGCT
TTCTCATAGCTACGCTGAGGTATCTCAGTTCGGTGTAGTCCGTTCCGCTCAAGCTGGGCTGTGTGCAC
GAACCCCGCTCAGCCCGACCGCTGCGCTTATCCGTTAACTATCGTCTTGAGTCCAAACCCGGTAAAGAC
ACGACTTATCGCCACTGGCAGCAGCCACTGGTAAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTAC
AGAGTTCTTGAAAGTGTGGCTAACTACGGCTACACTAGAAGCACGTATTTGGTATCTGCGCTCTGCTG
AAGCCAGTTACCTTCGGAAACAGAGTTGGTAGCTCTTGATCCGGCAACAAACCCGCTGTTAGCGGTG
GTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTC
TACGGGCTCTGACGCTCAGTGAACGAAACCTCACGTTAAGGATTTTGGTCATGAGATTATCAAAAAGG
ATCTTCACCTAGATCCTTTTAAATTAATAATGAAGTTTAAATCAATCTAAAGTATATATAGTAAACTT
GGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTTCGTTTCATCCAT
AGTTGCCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGCTTACCATCTGGCCCCAGTGTGCA
ATGATACCGGAGACCCACGCTCACCGGCTCCAGATTATCAGCAATAAACAGCAGCCAGCGAAGGCCG
AGCGCAGAAAGTGTCTGCAACTTTATCCGCTCCATCCAGTCTATTAAATTGTTGCCGGAAGCTAGAGT
AAGTAGTTCCGCCAGTTAATAGTTTGCACAACGTTGTTGCCATTGCTACAGGCATCGTGTGTCAAGCTCG
TCGTTTGGTATGGCTTCATTCAGCTCCGTTCCCAACGATCAAGCGAGTACATGATCCCCCATGTTGT
GCAAAAAGCGGTTAGCTCCTTCGGTCCCTCCGATCGTTGTGAGAAAGTAAAGTGGCCGAGTGTATCACT
CATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCCATCCGTAAGATGCTTTTCTGTGACTGGT
GAGTACTCAACCAAGTCAATCTGAGAAATAGTGTATGCGGCGACCGAGTTGCTCTTGGCCGGTCAATAC
GGATAATAACCGGCCACATAGCAGAACTTTAAAGTGTCTCATCATTTGGAAAACGTTCTTCGGGCGGAAA
ACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAAACCCACTCGTGCAACCAACTGATCTTCA
GCATCTTTTACTTTACCCAGCGTTTCTGGGTGAGCAAAAACAGGAAGCAAAATGCCCAAAAAGGGAA
TAAGGGCGACACGGAAATGTTGAATACTCATCTCTCTTTTCAATATATTGAAGCATTTATCAGGG
TTATTGTCTCATGAGCGGATACATATTTGAAATGTATTTAGAAAAATAACAAATAGGGGTTCCCGGCACA

C

12/25

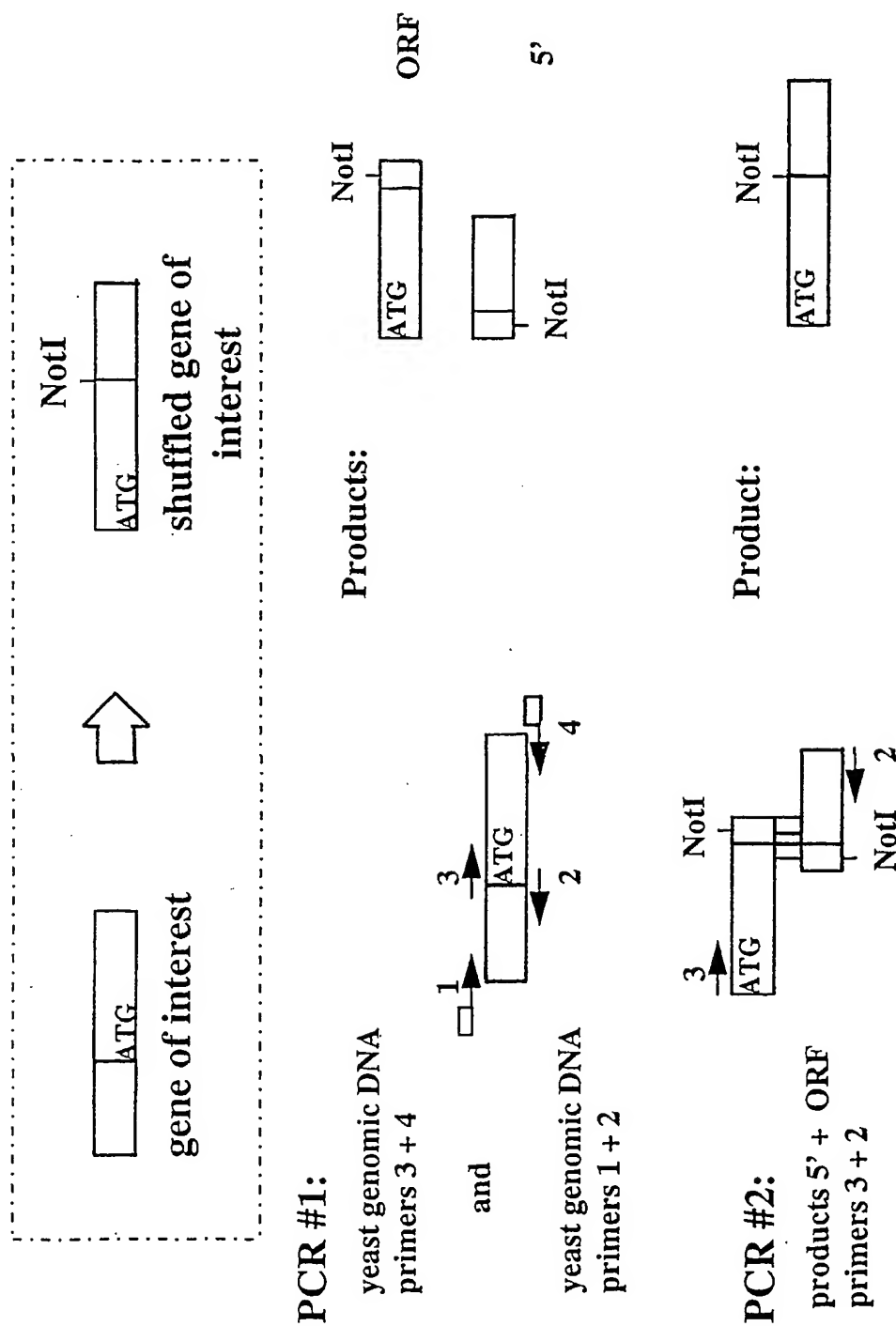
FIG. 5D

C

TTTCCCGAAAAGTGCCACCTGCTAAGAAACCATTTATTATCATGACATTAACTATATAAAATAGGCGTAT
CACGAGCCAGCTTTTCAATTCAATTCAATCATCATTTTTTTTTTTTATTCTTTTGTGATTTTCGGTTTCTTTGA
AATTTTGTGATTCGGTAAATCTCCGAACAGAAAGAAACGAAGAGACACAGACTTAGATTGGTAT
ATATACGCATATGTAGTGTGAAGAAACATGAATTGCCAGTATTCTTAACTTAACTGACACAGAAACAAA
AACATGCAGGAAACGAAGATAAATCATGTGAAAGCTACATATAAGGAAACGTGCTGCTACTCATCCTAGT
CCTGTTGCTGCCAAGCTATTTAATATCATGCAGAAAGCAAACTTGTGTGCTTTCATTTGGATGTTTC
GTACCAACCAAGGAATTACTGGAGTTAGTTGAAGCATTAGGTCCCAAAATTTGTTTACTATAAAACACATGT
GGATATCTTGACTGATTTTTCCTATGGAGGACAGTTAAGCCGCTAAAGGCATTATCCGCCAAGTACAAT
TTTTTACTCTTCGAAGACAGAAAATTTGCTGACATTGGTAATACAGTCAAAATTGCAGTACTCTGCGGGTG
TATACAGAAATAGCAGAAATGGCAGACATTACGAATGCACACGGTGTGGTGGCCAGGTATTGTTAGCGG
TTTGAAGCAGGCGGCAGAAAGTAACAAAGGAACCTAGAGGCCCTTTTGATGTTAGCAGAAATTGTCAATGC
AAGGGCTCCCTATCTACTGGAGAATATACTAAGGGTACTGTTGACATTGCGAAGAGCGACAAAGATTTTG
TTATCGGCTTTATTGCTCAAAGAGACATGGGTGGAAGAGATGAAGGTTACGATTGGTTGATTATGACACC
CGTGTGGGTTTAGATGACAAGGAGACGCATTGGGTCAACAGTATAGAACCCTGGATGATGTTGCTCT
ACAGGATCTGACATTATTATTGTTGGAAGAGGACTATTGCAAGGGAAGGATGCTAAGGTAGAGGGTG
AACGTTACAGAAAAGCAGGCTGGGAAGCATATTGAGAAGATGCGGCCAGCAAACTAAAAAATGTATT
ATAAGTAAATGCATGTATATACTAACTACAAAATTAGAGCTTCAATTTAATTATATCAGTTATTACCCGCC
CTTTCTGTCGCGCGTTTCGGTGATGACCGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACA
GCTTGTCTGTAAAGCGGATGCCGGAGCAGACAAAGCCGTCAGGGCGCGTCAGCGGTTGCGGGGTGTC
GGGCTGGCTTAACATATGCGGCATCAGAGCAGATTGTACTGAGAGTGCCACCATATGCGGTGTGAAATACC
GCACAGATGCGTAAGGAGAAAATACCGCATCAGGCGCCATTGCGCATTCAGGCTGCGCAACTGTGTTGGAA
GGCGATCGGTGCGGCGCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGATGTGCTGCAAGGCGATTAA
GTTGGGTAAACGCCAGGGTTTTCACAGTACGACGTTGTAAAAACGACGGCCAGT

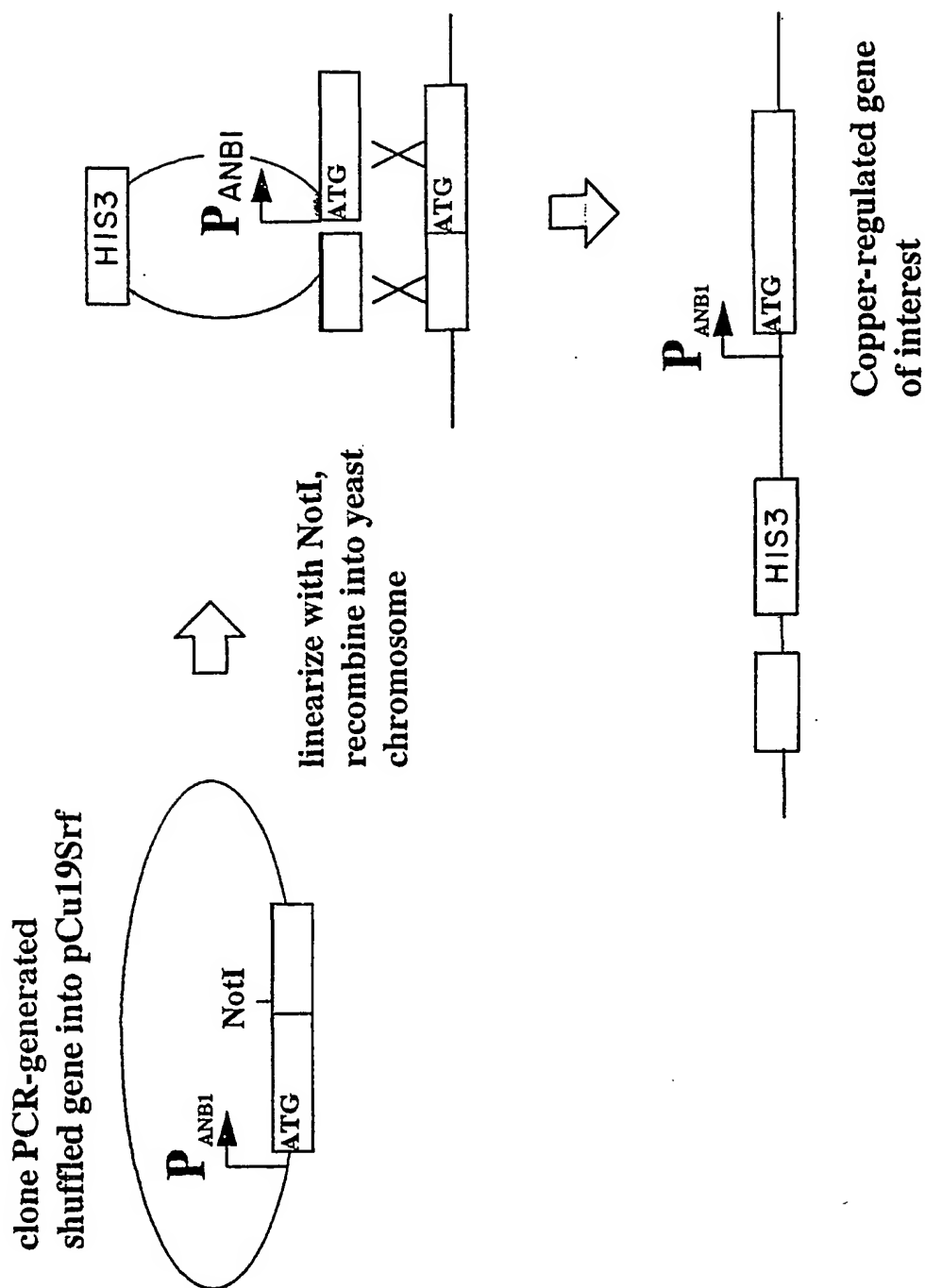
13/25

FIG. 6



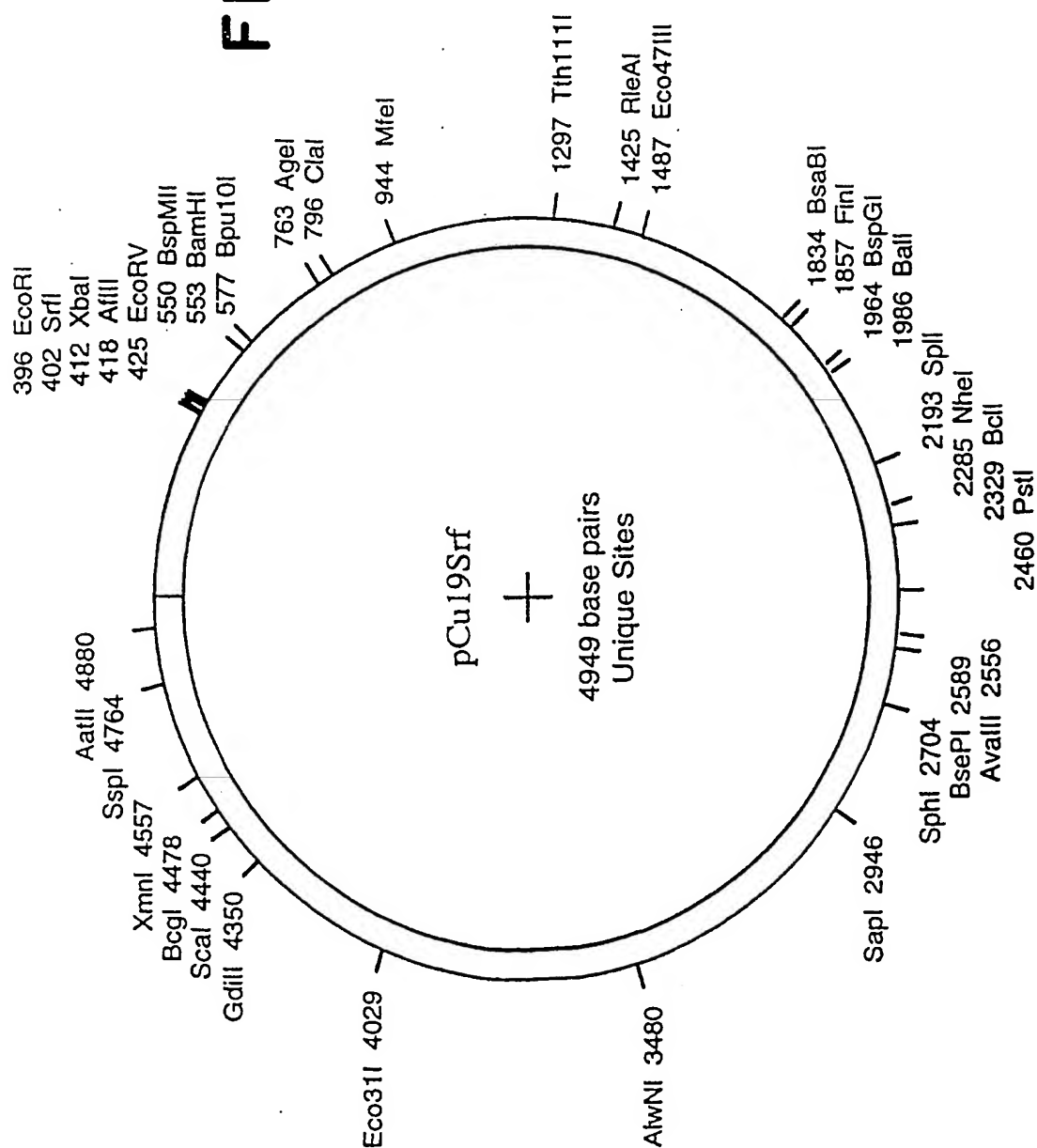
14/25

FIG. 7



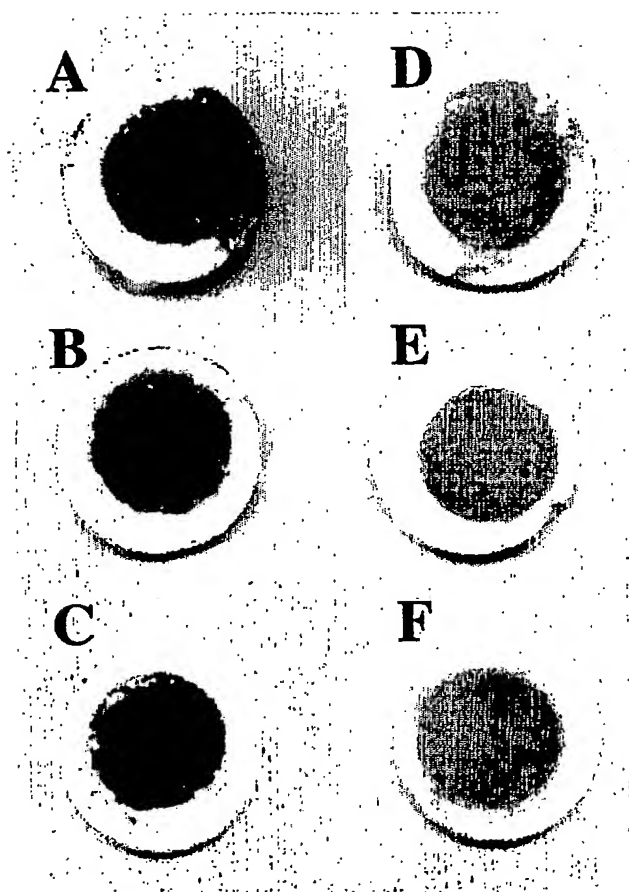
15/25

FIG. 8



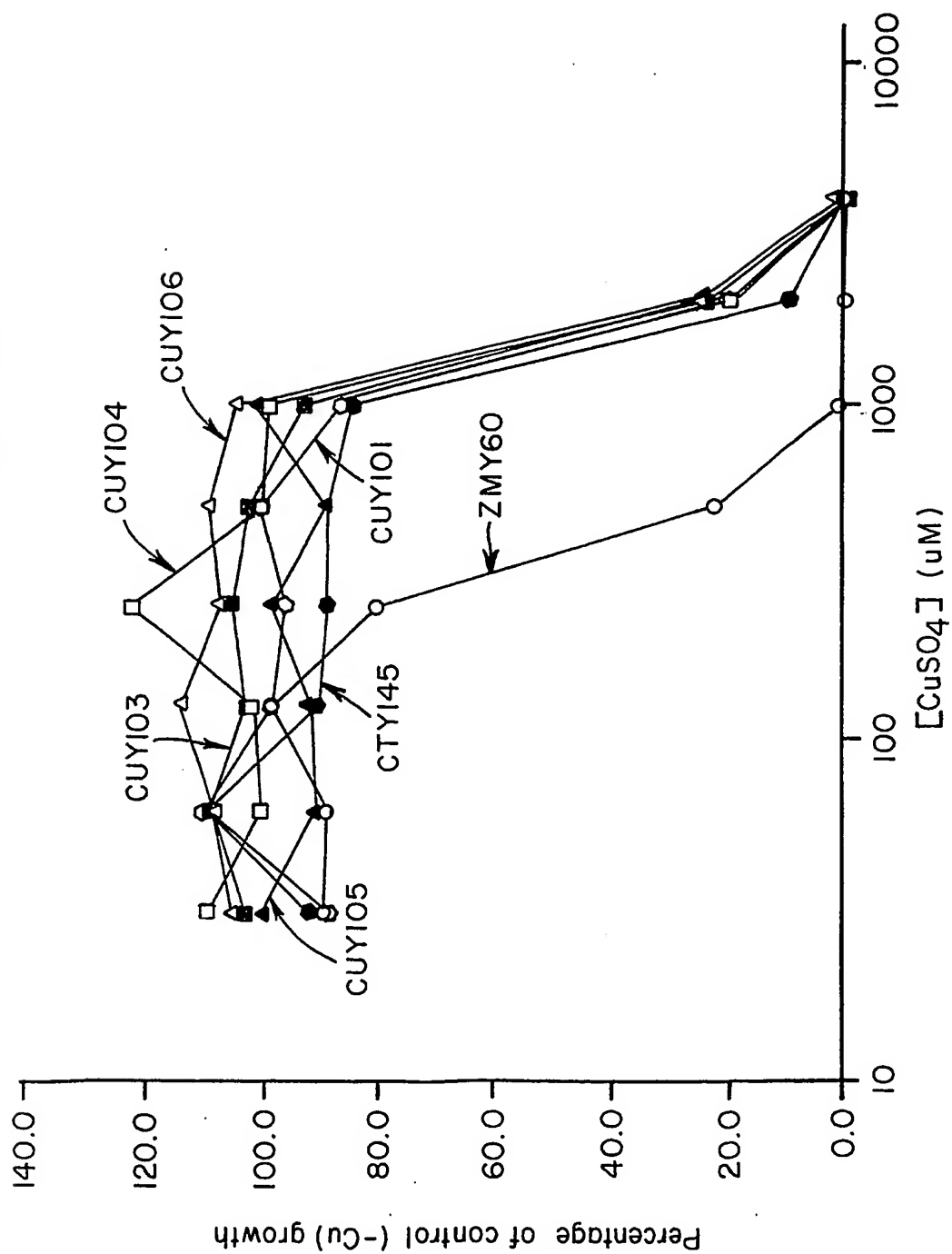
16/25

FIG. 9



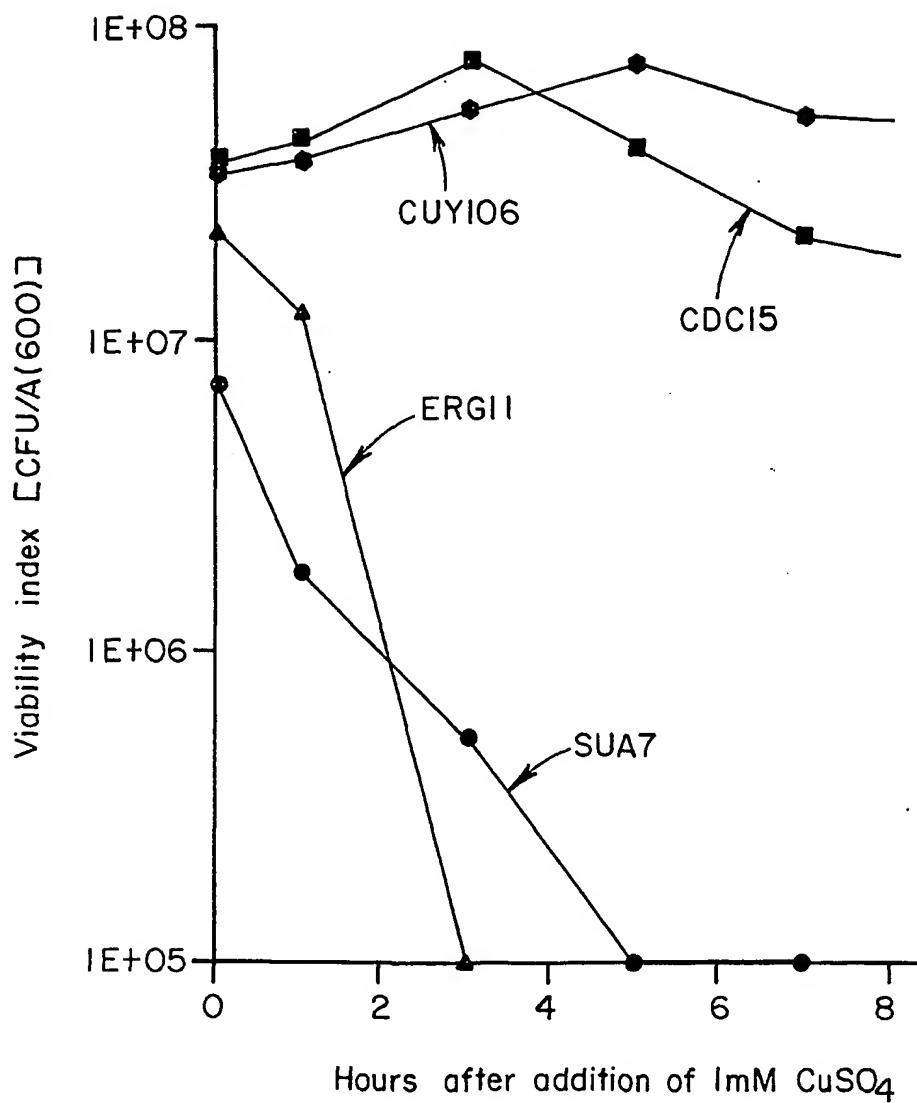
17/25

FIG. 10



18/25

FIG. 11



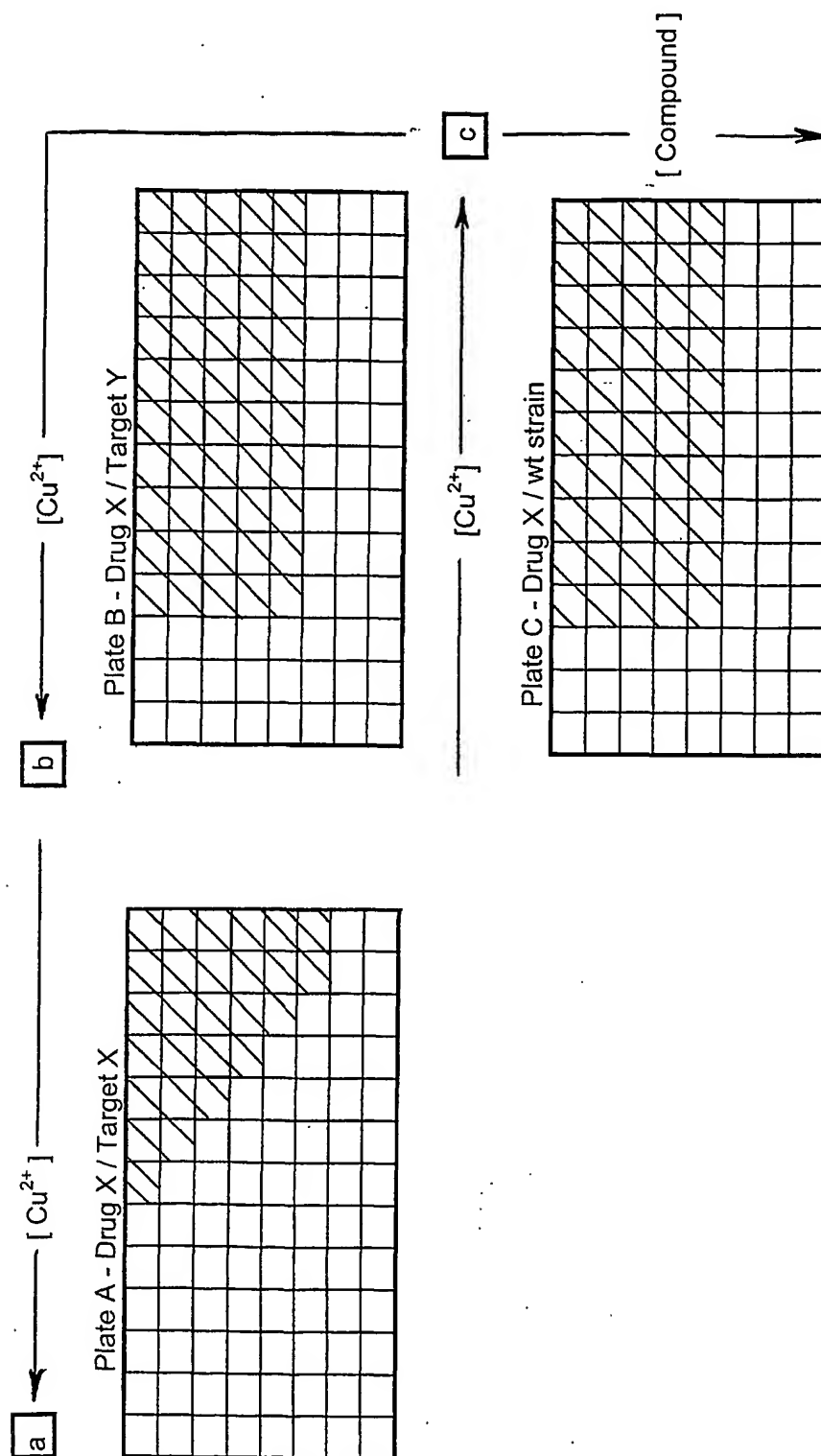
19/25

FIG. 12

μ g/ml Compound	mM copper sulfate												No Cu
	Cu/Drug	2	1	0.5	.25	0.125	0.063	0.032	0.016	0.008	0.004	0.0004	
0													
0.01													
0.1													
0.5													
1													
2													
8													
24													

20/25

FIG. 13

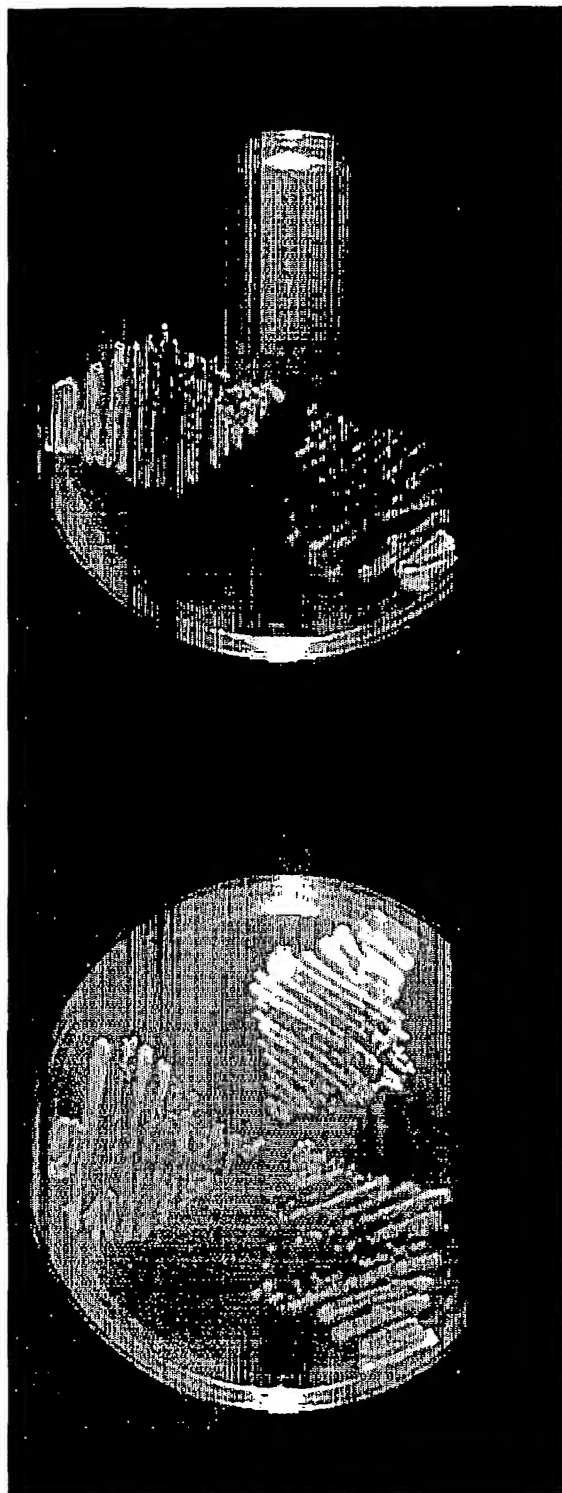


21/25

FIG. 14

+CuSO₄
BOS1

-CuSO₄
BOS1



vector

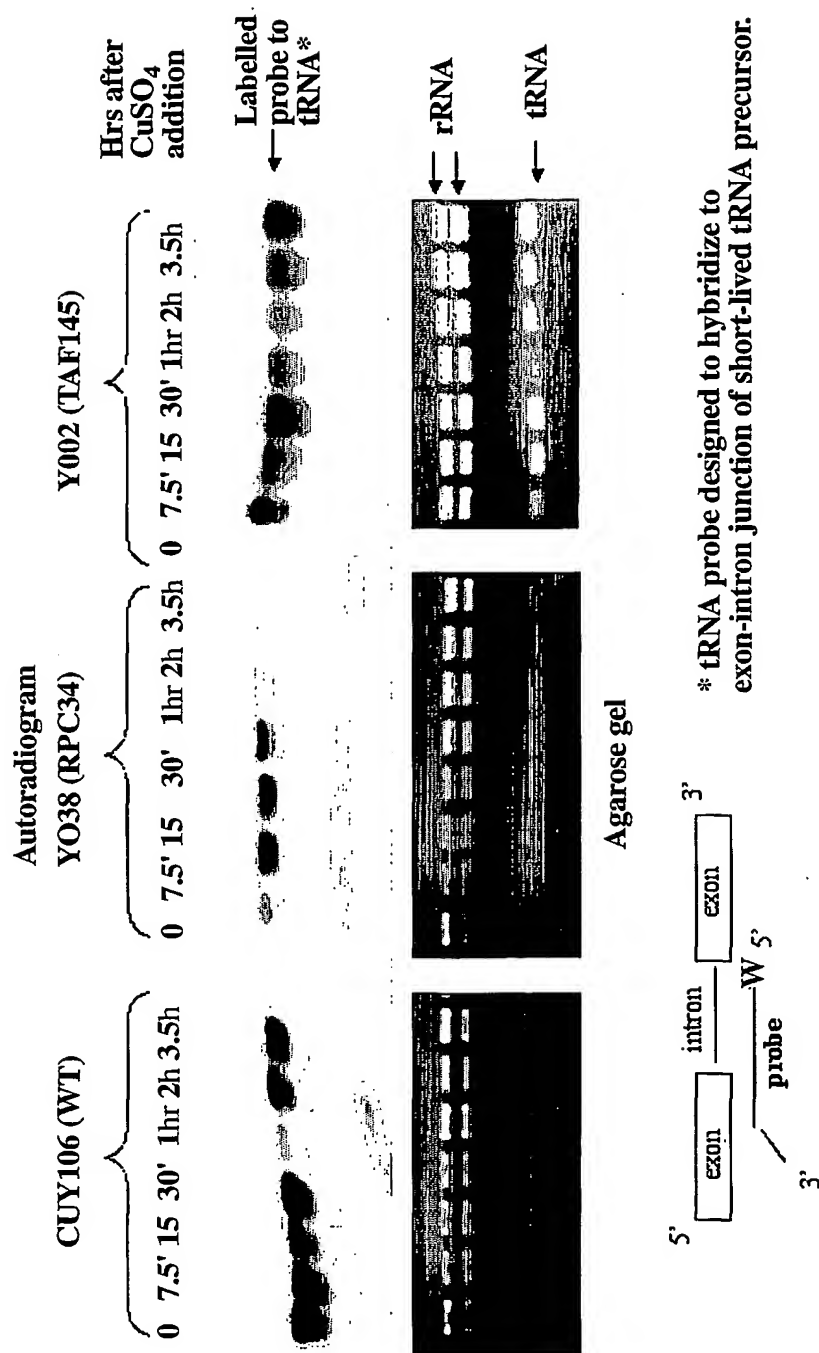
bos1-200

vector

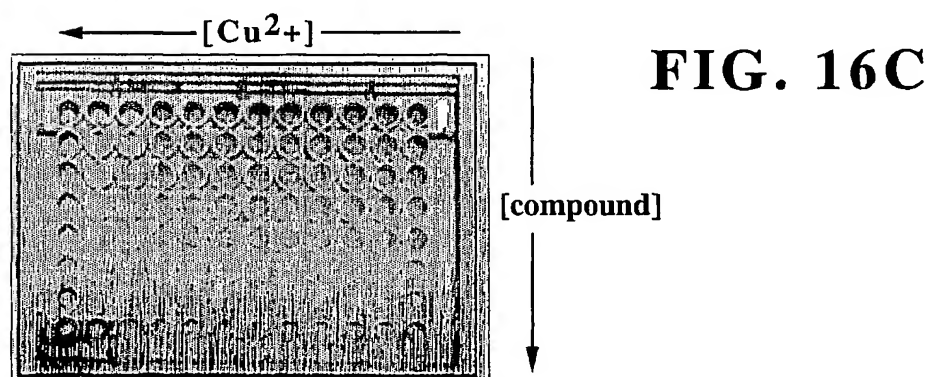
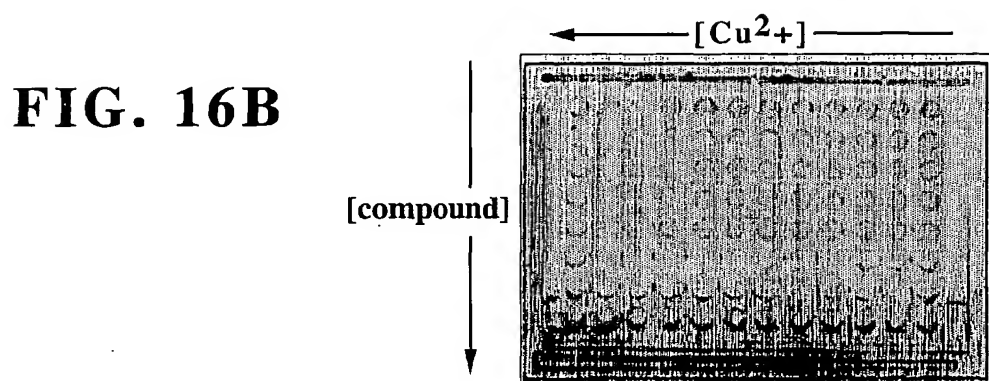
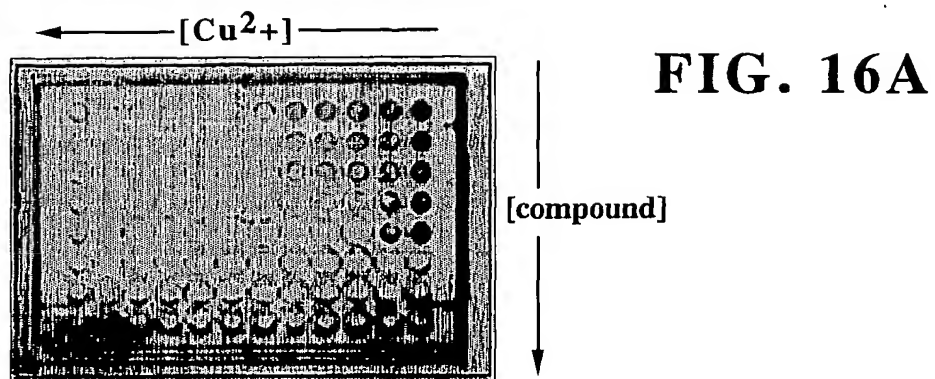
bos1 -200

22/25

FIG. 15



23/25



24/25

FIG. 17A

[Conc. µg]	2	1	0.5	0.2	0.1	0.05	0.02	0.01	0.005	0.002	0.001	No
	5	25	63	32	16	08	04	02	01	00	4	Co
0												
0.01												
0.1												
0.5												
1												
2												
8												
24												
Expt=	cu ERG11 vs Difluconazole											

FIG. 17B

[Conc. µg]	2	1	0.5	0.2	0.1	0.05	0.02	0.01	0.005	0.002	0.001	No
	5	25	63	32	16	08	04	02	01	00	4	Co
0												
0.01												
0.1												
0.5												
1												
2												
8												
24												
Expt=	Cu TUB1 vs Benomyl											

25/25

FIG. 17C

[Cu]/[Drug]	2	1	0.5	0.2	0.1	0.05	0.03	0.016	0.008	0.004	0.002	No Cu
0												
0.01												
0.1												
0.5												
1												
2												
8												
24												
Expt=	CuCMT vs Fluphenazine											

FIG. 17D

[Cu]/[Drug]	2	1	0.5	0.2	0.1	0.05	0.03	0.016	0.008	0.004	0.002	No Cu
0												
0.01												
0.1												
0.5												
1												
2												
8												
24												
Expt=	CuALG vs Tunicamycin											

SEQUENCE LISTING

<110> Bradley, John D.
 Thompson, Craig M.
 Moore, Jeffrey B.
 Wobbe, C. Richard
 Bailey, David A.

<120> Regulated Gene Expression in Yeast and
 Methods of Use

<130> 0342/1D469-US4

<140> 09/573,322

<141> 2000-05-18

<150> 09/404,066

<151> 1999-09-23

<150> 09/138,024

<151> 1998-08-21

<150> 60/056,719

<151> 1997-08-22

<160> 30

<170> FastSEQ for Windows Version 3.0

<210> 1

<211> 19

<212> DNA

<213> Artificial Sequence

<220>

<223> LexA operator

<400> 1

tactgatgta catacagta

19

<210> 2

<211> 52

<212> DNA

<213> Artificial Sequence

<220>

<223> Artificial LexA operator

<400> 2

tcgagtactg tatgtacata cagtaccatg acatacatgt atgtcatgag ct

52

<210> 3
<211> 42
<212> DNA
<213> ACE1 binding siteArtificial Sequence

<220>
<223> ACE1 binding site

<400> 3
taagtctttt ttgctggaac gggtgagcgg aaaagacgca tc 42

<210> 4
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> ROX-A PCR primer

<400> 4
tcacacaaaa gaacgcag 18

<210> 5
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> ROX-B PCR primer

<400> 5
gatgacagct gtggtagg 18

<210> 6
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> ROX-C PCR primer

<400> 6
tcttgccata tggatctg 18

<210> 7
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> UBR-A PCR primer

<400> 7
atcttcggac aaaggcag 18

<210> 8
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> UBR-B PCR primer

<400> 8
gtgtaatttt cgggatcg 18

<210> 9
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> ROX-C PCR Primer

<400> 9
tcttgccata tggatctg 18

<210> 10
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> SLF-E PCR primer

<400> 10
gcgctgcagg tcgacttagc aggcagtttg aac 33

<210> 11
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> SLF-F PCR primer

<400> 11
gcgctgcagg catgcactcc tttccaattg tgc 33

<210> 12
<211> 32
<212> DNA
<213> Artificial Sequence

<220>

<223> SLF-G PCR primer

<400> 12

gcgagctcgg taccccatatc ccctaactct ag

32

<210> 13

<211> 32

<212> DNA

<213> Artificial Sequence

<220>

<223> SLF-H PCR primer

<400> 13

gcggatcccg gggctctctc gtttatttaa cg

32

<210> 14

<211> 18

<212> DNA

<213> Artificial Sequence

<220>

<223> HISGCH PCR primer

<400> 14

gatttggtct ctaccggc

18

<210> 15

<211> 18

<212> DNA

<213> Artificial Sequence

<220>

<223> SLF-D PCR primer

<400> 15

gacagtatcg taattacg

18

<210> 16

<211> 21

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer 2b

<400> 16

ccagactacg cttcgatatc g

21

<210> 17

<211> 20

<212> DNA
 <213> Artificial Sequence

<220>
 <223> PCR primer 2a

<400> 17
 cacactaaaa catcgatatt 20

<210> 18
 <211> 20
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Universal HIS3-2STEP PCR primer

<400> 18
 caggcatgca agcttggcgt 20

<210> 19
 <211> 34
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Primer C typical sequence

<220>
 <221> unsure
 <222> (28)...(34)
 <223> n is a or g or c or t/u

<400> 19
 acaaatacac acactaaatt aataatgnnn nnnn 34

<210> 20
 <211> 7102
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> ZM195 plasmid

<400> 20
 gaattaattc gagctcggtc cgggtgatct tcgctcggcc acaaatcccc tggatatcat 60
 tggcctgtcg aggtatcggc cgcgtggaac taccggaat tactatgcaa aacaattgga 120
 aatctggtag gaaaaccttg ttctagaact tggcgattgc tgacaaagaa gaaaagggcc 180
 tattgttgct gcctcttttg ttgttcttcc tcgtattgtc ttgccggtgt tctttgtgtc 240
 ttttgtgtgt aggttcttac tattatagtg ctctttgcta ttatatatttc ttcgttttca 300
 ctttgcgtaa tgtaacggtc ttaaacaag tttttttttt ttcgctcttg cattttcctt 360
 ttctgctcta tcttatttgc taattgtagt ttcagaagtt ttaccttaaa tatagcacta 420

ttttccagtt	ttaatgtttc	ttctcattgc	tttcttttat	aatttttcgca	tataattata	480
cattttacggt	gtcttaactc	tccctcttca	cccctcatta	ttccagaaaa	tactaatact	540
tcttcacaca	aaagaacgca	gttagacaat	caacaatgac	tagtagtttt	tcttgaacca	600
aagaaagggtc	accagaggca	atagactcct	caatctcatt	gattctttgc	ttggcttctg	660
cagtggacga	gaacttggcc	tttttgccta	acttctcctc	aatttggttg	ttttttctct	720
tgatttgagc	atccaattgc	ttaatagagt	cgtgaatgtt	gcttctacgg	gttttcaagt	780
cagcttggat	cttgatgata	tccttgttct	tatcctgtaa	cttcttacgt	tcttgttggg	840
tggtatcggt	gacctggtgt	tgatcgattt	gctttctaata	taaaccgatt	tcagtgtcga	900
tttttttcaa	ttgaacgtta	agagtgtcca	atttcttgct	tctaaccggag	acatctgggc	960
gcttgaactt	gtgttggttg	gaggacatgg	caatggctgt	gttgtagaa	aatatgctat	1020
tacgttgata	aaaggaggaa	aggtgaaatc	agttcaaaaa	tgtgaatgaa	actgaacgaa	1080
gaaatgacca	gaatgagtga	aaaatggaga	tggaggggca	aaatgaaaaa	aaaaaaaagg	1140
atgaacctaa	aatagaaaat	agactccgtc	gtactttaat	gctatgtata	acgcaaccaa	1200
gcaattttcg	aaactcaatt	tggcttataa	atgttcgaga	taaaatgcga	attacgtgtt	1260
caacgtcgct	gagatcagtt	attttttttc	acgccacagt	gcgggtaagc	aatttttcgc	1320
gtaccaccac	cattacacat	gtataatgta	tataggctta	ttatgtatgt	ttgtgctact	1380
ttatatgacg	gttatttaca	agttagaata	ttatctatta	acaatgcagt	agccacgctt	1440
acgttttagtg	agtcaacaat	gggttctggg	gcccagattgc	ctttctcaat	gccaccaaag	1500
ggaatttcga	cgaagaagtc	actcctcatc	ttcaaattcg	ttcttacgcc	ctggctttcg	1560
ttccccacca	ctagaacaac	aggcagctcg	ttacataatc	cgttcaaatac	gtgcatgcta	1620
atagtttttc	caacagtgtg	tttttctgac	gtggcattag	ctaagtggct	tgtaataaac	1680
gtccagccac	ccatttcttg	tgatttagta	aaaaactcta	acggtttatc	aacgtaaaat	1740
atgggcagaa	gttcgagggc	cccactgctt	gtccttgaca	ccacaggcgt	caaaggagag	1800
cagtttcttc	tcgacatcac	aatgaagtca	acccccagga	agtaagcgct	tctaataatg	1860
gcaccgatat	tgtgagggtc	agttatttca	tccagatata	acccgagagg	aaacttctta	1920
gcgtctgttt	tcgtaccata	aggcagttca	tgaggatatat	tttcggttatt	gaagcccagc	1980
tcgtgaatgc	ttaatgctgc	tgaactgggtg	tccatgtcgc	ctagggtacgc	aatctccaca	2040
ggctgcaaag	gttttgtctc	aagagcaatg	ttattgtgca	ccccgtaatt	ggtcaacaag	2100
tttaatctgt	gcttgctcac	cagctctgtc	gtaaccttca	gttcacgcac	tatctgaaga	2160
aattttactag	gaatagtgc	atggtacagc	aaccgagaat	ggcaatttct	actcgggttc	2220
agcaacgctg	cataaacgct	gttggtgccg	tagacatatt	cgaagatagg	attatcattc	2280
ataagtttca	gagcaatgtc	cttattctgg	aacttggtat	tatggctctt	ttggtttaat	2340
ttcgccctgat	tcttgatctc	cttttagcttc	tcgacgtggg	cctttttctt	gccatatgga	2400
tctgaattct	agtctttttt	gctggaacgg	ttgagcggaa	aagacgcata	gaattcgagc	2460
tcgttagcga	ttggcattat	cacataatga	attatacatt	atataaagta	atgtgatttc	2520
ttcgaagaat	atactaaaaa	atgagcaggc	aagataaacg	aaggcaaagg	acggtatcga	2580
tatcaatgaa	tcctaaatcc	tctacaccta	agattccaag	acccaagaac	gcatttattc	2640
tgttcagaca	gcactaccac	aggatcttaa	tagacgaatg	gaccgctcaa	ggtgtggaaa	2700
taccccataa	ttcaaacatt	tctaaaatta	ttggtacgaa	gtggaagggc	ttacaaccgg	2760
aagataaggc	acactgggaa	aatctagcgg	agaaggagaa	actagaacat	gaaaggaagt	2820
atcctgaata	caaatacaag	ccggtgaagaa	agtctaagaa	gaagcaacta	cttttgaagg	2880
aaatcgagca	acagcagcag	caacaacaga	aagaacagca	gcagcagaaa	cagtcacaac	2940
cgcaattaca	acagcccttt	aacaacaata	tagttcttat	gaaaagagca	cattctcttt	3000
caccatcttc	ctcgggtgtca	agctcgaaca	gctatcagtt	ccaattgaac	aatgatctta	3060
agaggttgcc	tattccttct	gttaataactt	ctaactatat	ggtctccaga	tcctctagag	3120
tcgacctgca	ggcatgcaag	cttggcgtaa	tcatggtcat	agctgtttcc	tgtgtgaaat	3180
tgttatccgc	tcacaattcc	acacaacata	cgagccggaa	gcataaagtg	taaagcctgg	3240
ggtgcctaata	gagtgaagct	actcacatta	attgcgttgc	gctcactgcc	cgctttccag	3300
tcgggaaacc	tgtcgtgcca	gggggggatcc	actagttcta	gagtcgaccg	gcatgcaagc	3360
ttggcgtaata	catggtcata	gctgtttcct	gtgtgaaatt	gttatccgct	cacaattcca	3420
cacaacatac	gagccggaag	cataaagtgt	aaagcctggg	gtgcctaata	agtgaagctaa	3480
ctcacattaa	ttgcgttgcg	ctcactgccc	gctttccagt	cgggaaacct	gtcgtgccag	3540

ctgcattaat	gaatcggcc	acgcgcggg	agaggcggtt	tgcgtattgg	gcgctcttcc	3600
gcttcctcgc	tcactgactc	gctgcgctcg	gtcgttcggc	tgcggcgagc	ggtatcagct	3660
cactcaaagg	cggtaatacg	gttatccaca	gaatcagggg	ataacgcagg	aaagaacatg	3720
tgagcaaaag	gccagcaaaa	ggccaggaac	cgtaaaaagg	ccgcgttgct	ggcgtttttc	3780
cataggctcc	gccccctga	cgagcatcac	aaaaatcgac	gctcaagtca	gaggtggcga	3840
aacccgacag	gactataaag	ataccaggcg	tttccccctg	gaagctccct	cgtgcgctct	3900
cctgtttccga	ccctgccgct	taccggatac	ctgtccgcct	ttctcccttc	gggaagcgtg	3960
gcgcttttctc	atagctcacg	ctgtaggtat	ctcagttcgg	tgtaggtcgt	tcgctccaag	4020
ctgggctgtg	tgcacgaacc	ccccgttcag	cccgaaccgt	gcgccttata	cggtaactat	4080
cgtcttgagt	ccaacccggt	aagacacgac	ttatcgccac	tggcagcagc	cactggtaac	4140
aggattagca	gagcgaggta	tgtaggcgg	gctacagagt	tcttgaagtg	gtggcctaac	4200
tacggctaca	ctagaaggac	agtatttgg	atctgcgctc	tgctgaagcc	agttaccttc	4260
ggaaaaagag	ttggtagctc	ttgatccggc	aaacaaacca	ccgctggtag	cgggtggttt	4320
tttgtttgca	agcagcagat	tacgcgcaga	aaaaaaggat	ctcaagaaga	tcctttgatc	4380
ttttctacgg	ggtctgacgc	tcagtggaa	gaaaactcac	gttaagggat	tttggtcag	4440
agattatcaa	aaaggatctt	cacctagatc	cttttaaatt	aaaaatgaag	ttttaaatca	4500
atctaaagta	tatatgagta	aacttggctc	gacagttacc	aatgcttaat	cagtgaggca	4560
cctatctcag	cgatctgtct	atttcgttca	ttccatagttg	cctgactccc	cgtcgtgtag	4620
ataactacga	tacgggagg	cttaccatct	ggccccagtg	ctgcaatgat	accgcgagac	4680
ccacgctcac	cggctccaga	tttatcagca	ataaaccagc	cagccggaag	ggccgagcgc	4740
agaagtggtc	ctgcaacttt	atccgcctcc	atccagtcta	ttaattgttg	ccgggaagct	4800
agagtaagta	gttcgccagt	taatagtttg	cgcaacgttg	ttgccattgc	tacaggcatc	4860
gtggtgtcac	gctcgtcggt	tggtaggtg	tcattcagct	ccggttccca	acgatcaagg	4920
cgagttacat	gatcccccat	gttgtgcaaa	aaagcggtta	gctccttcgg	tcctccgatc	4980
gttgtcagaa	gtaagtggc	cgcagtgta	tcactcatgg	ttatggcagc	actgcataat	5040
tctcttactg	tcatgccatc	cgtaagatgc	ttttctgtga	ctggtgagta	ctcaaccaag	5100
tcattctgag	aatagtgtat	gcggcgaccg	agttgctctt	gcccggcgctc	aatacgggat	5160
aataccgcgc	cacatagcag	aactttaaaa	gtgctcatca	ttggaaaacg	ttcttcgggg	5220
cgaaaactct	caaggatctt	accgctgttg	agatccagtt	cgatgtaacc	cactcgtgca	5280
cccaactgat	cttcagcatc	ttttactttc	accagcgttt	ctgggtgagc	aaaaacagga	5340
aggcaaaatg	ccgcaaaaaa	gggaataagg	gcgacacgga	aatggtgaat	actcatactc	5400
ttcctttttc	aatattattg	aagcatttat	cagggttatt	gtctcatgag	cggatacata	5460
tttgaatgta	tttagaaaaa	taaacaaata	ggggttcgcg	gcacattttc	ccgaaaagtg	5520
ccacctgacg	tctaagaaac	cattattatc	atgacattaa	cctataaaaa	taggcgtatc	5580
acgaggccag	cttttcaatt	caattcatca	tttttttttt	attctttttt	ttgatttcgg	5640
tttctttgaa	atttttttga	ttcggtaatc	tccgaacaga	aggaagaacg	aaggaaggag	5700
cacagactta	gattggtata	tatacgcata	tgtagtgttg	aagaaacatg	aaattgccca	5760
gtattcttaa	cccaactgca	cagaacaaaa	acatgcagga	aacgaagata	aatcatgtcg	5820
aaagctacat	ataaggaacg	tgctgctact	catcctagtc	ctggtgctgc	caagctattt	5880
aatatcatgc	acgaaaagca	aacaaaactg	tgtgcttcat	tggatgttcg	taccaccaag	5940
gaattactgg	agttagtgtga	agcattaggt	cccaaaattt	gtttactaaa	aacacatgtg	6000
gatatacttg	ctgatttttc	catggagggc	acagttaagc	cgctaaaggc	attatccgcc	6060
aagtacaatt	ttttactctt	cgaagacaga	aaatttgctg	acattggtaa	tacagtcaaa	6120
ttgcagtact	ctgcgggtgt	atacagaata	gcagaatggg	cagacattac	gaatgcacac	6180
ggtgtggtgg	gccaggtat	tgttagcgg	ttgaagcagg	cggcagaaga	agtaacaaag	6240
gaacctagag	gccttttgat	gttagcagaa	ttgtcatgca	agggtccct	atctactgga	6300
gaatatacta	agggtagtgt	tgacattg	aagagcgaca	aagattttgt	tatcggcttt	6360
attgctcaaa	gagacatggg	tgggaagag	gaaggttacg	attggttgat	tatgacaccc	6420
ggtgtgggtt	tagatgacaa	gggagacgca	ttgggtcaac	agtatagaac	cgtggatgat	6480
gtggtctcta	caggatctga	cattattatt	gttgggaag	gactatttgc	aaagggaagg	6540
gatgctaagg	tagagggtga	acgttacaga	aaagcaggct	gggaagcata	tttgagaaga	6600
tgcgggccagc	aaaactaaaa	aactgtatta	taagtaaag	catgtatact	aaactcacia	6660

attagagctt	caattttaatt	atatcagtta	ttacccgcc	tttcgtctcg	cgcgtttcg	6720
tgatgacggt	gaaaacctct	gacacatgca	gctcccgag	acggtcacag	cttgtctgta	6780
agcggatgcc	gggagcagac	aagcccgta	gggcgcgta	gcgggtgttg	gcgggtgtcg	6840
gggctggctt	aactatgcgg	catcagagca	gattgtactg	agagtgcacc	atatgcgggtg	6900
tgaaataccg	cacagatgcg	taaggagaaa	ataccgcac	aggcgccatt	cgccattcag	6960
gctgcgcaac	tggttggaag	ggcgatcggt	gcgggcctct	tcgctattac	gccagctggc	7020
gaaaggggga	tgtgctgcaa	ggcgattaag	ttgggtaacg	ccagggtttt	cccagtcacg	7080
acgttgtaaa	acgacggcca	gt				7102

<210> 21

<211> 7333

<212> DNA

<213> Artificial Sequence

<220>

<223> ZM197 plasmid

<400> 21

gaattaattc	gagctcggt	ccagttgcc	caccacaaaa	gtcgaaaaag	gctaagaaac	60
caaagaataa	ggtactaagt	accaggcg	tactaagacc	aacgagattg	ccacgaaact	120
agaggaaacc	aaattgtaag	catagcttaa	tccgttttca	cgattcataa	tataataaat	180
aagaaaagat	atatcatata	aacgttataa	aattaataac	cggttaagt	tagaaaagt	240
atgcgacggt	ttattttctc	ttcctcttgc	gattgaattt	aacttgcaga	tagtgaccat	300
aaggcaacta	cccagtgcca	aacagttttg	ataacgcca	gtacatcaac	gagcgagtat	360
aaagactttg	gtacatttta	aaaaggaaac	atatattgtt	ttcattgcta	gacctttta	420
gtctcacctc	aataaaactg	ctttattcct	cattgggctt	tttattcttt	aattttgcat	480
acttatagcg	tgaaactggg	catttaacaa	aagcaaacta	ttttaatagt	agcatcctgc	540
tttctttgcc	cctccttctt	attgcgatac	attattaagt	ttttttacca	cctttcttcc	600
ttttcttctg	catcttcgga	caaaggcagt	tgaagtttac	tgtatcctat	tagttgacta	660
ttttctctca	ctgaagtcct	taatctttac	aggtcacaca	aattacatag	aacattccaa	720
ctagtagttt	ttcttgaacc	aaagaaaggt	caccagagge	aatagactct	tcaatctcat	780
tgattctttg	cttggtctct	gcagtggaag	agaacttggc	ctttttgcct	aacttctcct	840
caatttggtt	gttttttctc	ttgatttgag	catccaattg	cttaatagag	tcgtgaatgt	900
tgcttctacg	ggttttcaag	tcagcttgga	tcttgatgat	ctccttggtc	ttatcctgta	960
acttcttacg	ttcttgttgg	gtggtatcgt	tgacctggtg	ttgatcgatt	tgctttctaa	1020
ttaaaccgat	ttcagtgctg	atttttttca	attgaacgtt	aagagtgtcc	aatttcttgt	1080
ctctaaccga	gacatctggg	cgcttgaact	tgtgttgttg	ggaggacatg	gcaatggctg	1140
tggtgttaga	aaatatgcta	ttacgttgat	aaaaggagga	aaggtgaaat	cagttcaaaa	1200
atgtgaatga	aactgaacga	agaaatgacc	agaatgagtg	aaaaatggag	atggaggggc	1260
aaaatgaaaa	aaaaaaaaag	gatgaacct	aaatagaaaa	tagactccgt	cgtactttta	1320
tgctatgtat	aacgcaacca	agcaattttc	gaaactcaat	ttggcttata	aatgttcgag	1380
ataaaatgcg	aattacgtgt	tcaacgtcgt	cgagatcagt	tatttttttt	cacgccacag	1440
tgcggttaag	caatttttctg	cgtaccacca	ccattacaca	tgtataatgt	atataggctt	1500
attatgtatg	tttgtgtctac	tttatatgac	ggttattttac	aagttagaat	attatctatt	1560
aacaatgcag	tagccacgct	tacgttttagt	gagtcaacaa	tggttcttgg	ggcccagattg	1620
cctttctcaa	tgccacccaa	gggaatttctg	acgaagaagt	cactcctcat	cttcaaatc	1680
gttcttacgc	cctggctttc	gttccccacc	actagaacaa	caggcagctc	gttacataat	1740
ccgttcaaat	cgtgcacgtc	aatagttttt	ccaacagtg	atttttctga	cgtggcatta	1800
gctaagtggc	ttgtaataaa	cgtccagcca	cccatttctt	gtgatttagt	aaaaaactct	1860
aacggtttat	caacgtaaaa	tatgggcaga	agttcgaggg	ccccactgct	tgtcttggac	1920

accacaggcg	tcaaaggaga	gcagttttctt	ctcgacatca	caatgaagtc	aacccccagg	1980
aagtaagcgc	ttctaataat	ggcaccgata	ttgtgagggt	cagttatttc	atccagatat	2040
aacccgagag	gaaacttctt	agcgtctgtt	ttcgtaccat	aaggcagttc	atgagggtata	2100
ttttcgttat	tgaagccag	ctcgtgaatg	cttaatgctg	ctgaactggg	gtccatgtcg	2160
cctaggtacg	caatctccac	aggctgcaaa	ggttttgtct	caagagcaat	gttattgtgc	2220
accccgtaat	tgggtcacaa	gtttaatctg	tgcttggtcca	ccagctctgt	cgtaaccttc	2280
agttcatcga	ctatctgaag	aaatttacta	ggaatagtcg	catggtacag	caaccgagaa	2340
tggcaatttc	tactcgggtt	cagcaacgct	gcataaacgc	tggttggtgcc	gtagacatat	2400
tcgaagatag	gattatcatt	cataagtttc	agagcaatgt	cottattctg	gaacttggat	2460
ttatggctct	tttgggttaa	tttcgcctga	ttcttgatct	cotttagctt	ctcgacgtgg	2520
gcctttttct	tgccatatgg	atctgaattc	tagtcttttt	tgctggaacg	gttgagcgga	2580
aaagacgcat	cgaattcgag	ctcgttagcg	attggcatta	tcacataatg	aattatacat	2640
tatataaagt	aatgtgattt	cttcgaagaa	tatactaaaa	aatgagcagg	caagataaac	2700
gaaggcaaag	gacgggtatcg	ataagcttgg	gaattcaaaa	tgcccaagaa	gaagcggaag	2760
gtccatatgt	acccatacga	cgttccagac	tacgcttctt	tgggtgggtc	tagcccaagc	2820
ttgatatcga	attcctgcag	cccgggggat	cctaacatgt	ccgttgctga	tgatgattta	2880
ggatctttac	aaggtcacat	taggagaaca	ctgaggtcta	ttcataacct	cccctatttt	2940
aggtatacga	gaggtcctac	tgaaagggct	gacatgagca	gagcccttaa	agagttcatt	3000
tacagatatc	tatactttgt	catttctaac	agcggagaga	acttacctac	tttattcaat	3060
gctcatccaa	aacaaaaaat	atctaacca	gagcttactg	tttttctctga	cagtttagaa	3120
gatgctgtgg	atattgataa	gataacatct	caacaaacta	ttccgtttta	taagatagat	3180
gaatccagaa	taggagacgt	ccataaacat	accggaagaa	attgtgggag	gaaattcaaa	3240
ataggggaac	ccttgatatag	gtgtcatgag	tgtgggtgog	atgatacttg	tgtgctttgt	3300
attcattgtt	ttaatccaaa	agatcatgtg	aatcatcatg	tttgtaccga	tatatgtact	3360
gaattcgata	tcaagcttgg	cgtaatcatg	gtcatagctg	tttcctgtgt	gaaattgtta	3420
tccgctcaca	attccacaca	acatacagac	cggaagcata	aagtgtaaaag	cctgggggtgc	3480
ctaattgagt	agctaactca	cattaattgc	gttgcgctca	ctgcccgtt	tccagtcggg	3540
aaacctgtcg	tgccaggggg	gatccactag	ttctagagtc	gaccggcatg	caagcttggc	3600
gtaatcatgg	tcatagctgt	ttcctgtgtg	aaattgttat	ccgctcacia	ttccacacaa	3660
catacagacc	ggaagcataa	agtgtaaaag	ctgggggtgcc	taatgagtga	gctaactcac	3720
attaattgcg	ttgcgctcac	tgcccgcctt	ccagtcggga	aacctgtcgt	gccagctgca	3780
ttaatgaatc	ggccaacgog	cggggagagg	cggtttgctg	attgggcgct	cttccgcttc	3840
ctcgctcact	gactcgctgc	gctcgggtcg	tcggctgogg	cgagcgggtat	cagctcactc	3900
aaaggcggta	atacggttat	ccacagaatc	aggggataac	gcaggaaaga	acatgtgagc	3960
aaaaggccag	caaaaggcca	ggaaccgtaa	aaaggccgog	ttgctggcgt	ttttccatag	4020
gctccgcccc	cctgacgagc	atcacaaaaa	tcgacgctca	agtcagaggt	ggcgaaaccc	4080
gacaggacta	taaagatacc	aggcgtttcc	ccctggaagc	tcctctgtgc	gctctcctgt	4140
tccgaccttg	ccgcttaccg	gatacctgtc	cgcctttctc	ccttcgggaa	gcgtggcgct	4200
ttctcatagc	tcacgctgta	ggtatctcag	ttcgggtgtg	gtcgttcgct	ccaagctggg	4260
ctgtgtgcac	gaaccccccg	ttcagccoga	ccgctgcgoc	ttatccggta	actatcgtct	4320
tgagtccaac	ccggtaaagc	acgacttatc	gccactggca	gcagccactg	gtaacaggat	4380
tagcagagcg	aggatatgtg	gcggtgctac	agagttcttg	aagtgggtggc	ctaactacgg	4440
ctacactaga	aggacagtat	ttggtatctg	cgctctgctg	aagccagtta	ccttcggaaa	4500
aagagttggt	agctcttgat	ccggcaaaaca	aaccaccgct	ggtagcgggtg	gtttttttgt	4560
ttgcaagcag	cagattacgc	gcagaaaaaa	aggatctcaa	gaagatcctt	tgatcttttc	4620
tacgggggtct	gacgctcagt	ggaacgaaaa	ctcacgttaa	gggatttttg	tcatgagatt	4680
atcaaaaagg	atcttcacct	agatcctttt	aaattaaqaa	tgaagtttta	aatcaatcta	4740
aagtatatat	gagtaaactt	ggtctgacag	ttaccaatgc	ttaatcagtg	aggcacctat	4800
ctcagcgatc	tgtctatttc	gttcatccat	agttgcctga	ctccccgtcg	tgtagataac	4860
tacgatacgg	gagggccttac	catctggccc	cagtgctgca	atgataccgc	gagacccacg	4920
ctcaccggct	ccagatttat	cagcaataaa	ccagccagoc	ggaagggccg	agcgcagaag	4980
tggctcctgca	actttatccg	cctccatcca	gtctattaat	tggtgccggg	aagctagagt	5040

aagtagttcg	ccagttaata	gtttgcgcaa	cgttgttgcc	attgctacag	gcatcgtggg	5100
gtcacgctcg	tcgtttggta	tggcttcatt	cagctccggg	tcccaacgat	caaggcgagt	5160
tacatgatcc	cccatgtttg	gcaaaaaagc	ggttagctcc	ttcggtcctc	cgatcgttgt	5220
cagaagtaag	ttggccgcag	tgttatcact	catggttatg	gcagcactgc	ataattctct	5280
tactgtcatg	ccatccgtaa	gatgcttttc	tgtgactggg	gagtactcaa	ccaagtcatt	5340
ctgagaatag	tgtatgcggc	gaccgagttg	ctcttgcccc	gcgtcaatac	gggataatac	5400
cgcgccacat	agcagaactt	taaaagtgtc	catcattgga	aaacgttctt	cggggcgaaa	5460
actctcaagg	atcttaccgc	tgttgagatc	cagttcgatg	taaccactc	gtgcacccaa	5520
ctgatcttca	gcatctttta	ctttcaccag	cgtttctggg	tgagcaaaaa	caggaaggca	5580
aaatgccgca	aaaaagggaa	taagggcgac	acggaaatgt	tgaatactca	tactcttcct	5640
ttttcaatat	tattgaagca	tttatcaggg	ttattgtctc	atgagcggat	acatatattga	5700
atgtatttag	aaaaataaac	aaataggggt	tccgcgcaca	tttccccgaa	aagtgccacc	5760
tgctaagaaa	ccattattat	catgacatta	acctataaaa	ataggcgtat	cacgaggcca	5820
gcttttcaat	tcaattcatc	atTTTTTTTT	tattcttttt	tttgatttcg	gtttctttga	5880
aatttttttg	attcggtaat	ctccgaacag	aaggaagaac	gaaggaagga	gcacagactt	5940
agattgggat	atatacgcat	atgtagtggt	gaagaaacat	gaaattgcc	agtattctta	6000
acccaactgc	acagaacaaa	aacatgcagg	aaacgaagat	aaatcatgtc	gaaagctaca	6060
tataaggaac	gtgctgctac	tcacctctagt	cctgttgctg	ccaagctatt	taatatcatg	6120
cacgaaaagc	aaacaaactt	gtgtgcttca	ttggatgttc	gtaccaccaa	ggaattactg	6180
gagttagttg	aagcattagg	tcccaaaatt	tgtttactaa	aaacacatgt	ggatatcttg	6240
actgattttt	ccatggaggg	cacagttaag	ccgctaaagg	cattatccgc	caagtacaat	6300
tttttactct	tcgaagacag	aaaatttgct	gacattggta	atacagtcaa	attgcagtac	6360
tctgcgggtg	tatacagaat	agcagaatgg	gcagacatta	cgaatgcaca	cgggtgtggtg	6420
ggcccaggta	ttgttagcgg	tttgaagcag	gcggcagaag	aagtaacaaa	ggaacctaga	6480
ggccttttga	tgtagcaga	attgtcatgc	aagggtccc	tatctactgg	agaatatact	6540
aagggtactg	ttgacattgc	gaagagcgac	aaagattttg	ttatcggtt	tattgctcaa	6600
agagacatgg	gtggaagaga	tgaagggttac	gattgggtga	ttatgacacc	cgggtgtgggt	6660
ttagatgaca	aggagacgc	attgggtcaa	cagtatagaa	ccgtggatga	tgtggtctct	6720
acaggatctg	acattattat	tgttggaaga	ggactatttg	caaaggggaag	ggatgctaag	6780
gtagagggtg	aacgttacag	aaaagcaggc	tgggaagcat	atttgagaag	atgcggccag	6840
caaaactaaa	aaactgtatt	ataagtaa	gcatgtatac	taaactcaca	aattagagct	6900
tcaattta	tatatcagtt	attaccgcc	ctttcgtctc	gcgcgtttcg	gtgatgacgg	6960
tgaaaacctc	tgacacatgc	agctcccggg	gacggtcaca	gcttgtctgt	aagcggatgc	7020
cgggagcaga	caagcccgtc	agggcgcgtc	agcgggtgtt	ggcgggtgtc	ggggctggct	7080
taactatgcg	gcatcagagc	agattgtact	gagagtgcac	catatgcggg	gtgaaatacc	7140
gcacagatgc	gtaaggagaa	aataccgcat	caggcgccat	tcgccattca	ggctgcgcaa	7200
ctgttgggaa	ggcgatcgg	tgcgggcctc	ttcgctatta	cgccagctgg	cgaaaggggg	7260
atgtgctgca	aggcgattaa	gttgggtaac	gccagggttt	tcccagtcac	gacgttgtaa	7320
aacgacggcc	agt					7333

<210> 22

<211> 4949

<212> DNA

<213> Artificial Sequence

<220>

<223> pCU19Srf vector

<400> 22

tcgcgcgttt cggtgatgac ggtgaaaacc tctgacacat gcagctcccg gagacgggtca 60

cagcttgtct	gtaagcggat	gccgggagca	gacaagcccg	tcagggcgcg	tcagcgggtg	120
ttggcgggtg	tcggggctgg	cttaactatg	cggcacatcaga	gcagattgta	ctgagagtgc	180
accatatgcg	gtgtgaaata	ccgcacagat	gcgtaaggag	aaaataccgc	atcaggcgcc	240
attcgccatt	caggctgcmc	aactgttggg	aagggcgatc	ggtgcgggccc	tcttcgctat	300
tacgccagct	ggcgaaaggg	ggatgtgctg	caaggcgatt	aagttgggta	acgccagggt	360
tttcccagtc	acgacgttgt	aaaacgacgg	ccagtgaatt	cgcccgggcg	atctagactt	420
aagcgatatc	gaagcgtagt	ctggaacgtc	gtatgggtag	gaatcggcca	acgcgcgggg	480
agaggcggtt	tgcgtattgg	gcgccagggt	ggtttttctt	ttcaccagt	agacgggcaa	540
cagccaagct	ccgatccgt	gcctaccacc	tcttagcctt	agcacaagat	gtaagggtga	600
ctccttctga	atgttgtaat	cagacagcgt	tctaccgtct	tctagctgct	taccggcaaa	660
gatcaatctt	tgttgatctg	gagggatacc	ttccttgtct	tgaattttcg	acttaacgtt	720
gtcgtatggt	tcggaagatt	caacttccaa	tgttatgggt	ttaccgggtca	aagtcttgac	780
gaaaatctgc	ataatatcga	tgttttagtg	tgtgaatgaa	ataggtgtat	gttttctttt	840
tgctagacaa	taattaggaa	caaggtaagg	gaactaaagt	gtagaataag	attaaaaaag	900
aagaacaagt	tgaaaaggca	agttgaaatt	tcaagaaaaa	agtcaattga	agtacagtaa	960
attgacctga	atatatctga	gttccgacaa	caatgagttt	accgaagaga	acaatggaat	020
aggaaacttt	gaacatagaa	aggaaagcag	gaaaggaaaa	aatttttagg	ctcgagaaca	080
atagggcaaa	aaaacaggca	acgaacgaac	aatggaaaaa	cgaaaaaaaa	aaaacacaga	140
aaagaatgca	gaaagtgtga	aactgaaaaa	aaaaaaaaaa	aggtgaacac	aggaaaaaaa	200
ataaaaaaaa	aaaaaaagga	ggacgaaaca	aaaaagtga	aaaaaatgaa	aatttttttg	260
gaaaaccaag	aatgaatta	tatttccgtg	tgagacgaca	tcgtcgaata	tgattcaggt	320
accgggctg	ttccctagca	tgtacgtgag	cgtatttccct	tttaaacac	gacgctttgt	380
cttcattcaa	cgtttcccat	tgtttttttc	tactattgct	ttgctgtggg	aaaaacttat	440
cgaaagatga	cgactttttc	ttaattctcg	ttttaagagc	ttggtgagcg	ctaggagtca	500
ctgccaggta	tcgtttgaac	acggcattag	tcagggaagt	cataacacag	tcctttccc	560
caattttctt	tttctattac	tcttggcctc	ctctagtaca	ctctatatatt	ttttatgcct	620
cggtaatgat	tttcattttt	ttttttccac	ctagcggatg	actctttttt	tttcttagcg	680
attggcatta	tcacataatg	aattatacat	tatataaagt	aatgtgattt	cttcgaagaa	740
tataactaaa	aatgagcagg	caagataaac	gaaggcaag	atgacagagc	agaaagccct	800
agtaaagcgt	attacaaatg	aaaccaagat	tcagattgcg	atctcttta	agggtgggtcc	860
cctagcgata	gagcactcga	tcttcccaga	aaaagaggca	gaagcagtag	cagaacaggc	920
cacacaatcg	caagtgatta	acgtccacac	aggtatagg	tttctggacc	atatgataca	980
tgctctggcc	aagcattccg	gctggctcgt	aatcggttag	tgcatgtgtg	acttacacat	040
agacgaccat	cacaccactg	aagactgcgg	gattgctctc	ggtcaagcct	ttaaagaggc	100
cctactggcg	cgtggagtaa	aaagggttgg	atcaggattt	gcgccttttg	atgaggcact	160
ttccagagcg	gtggtagatc	tttgcgaacg	gccgtacgca	gtgtgcgaac	ttggttgca	220
aaggagaaaa	gtaggagatc	tctcttgcg	gatgatcccg	cattttcttg	aaagctttgc	280
agaggctagc	agaattaccc	tccacgttga	ttgtctgcga	ggcaagaatg	atcatcaccc	340
tagtgagagt	gcgttcaagg	ctcttgccgt	tgccataaga	gaagccacct	cgcccaatgg	400
taccaacgat	gttccctcca	ccaaagggtg	tcttatgtag	tgacaccgat	tatttaaagc	460
tgcagcatac	gatatatata	catgtgtata	tatgtatacc	tatgaatgtc	agtaagtatg	520
tatacgaaca	gtatgatact	gaagatgaca	aggtaatgca	tcattctata	cgtgtcattc	580
tgaacgaggg	gcgctttcct	tttttctttt	tgctttttct	ttttttttct	cttgaactcg	640
agaaaaaaa	tataaaagag	atggaggaac	gggaaaaagt	tagttgtggg	gatagggtggc	700
aaggcatgca	agcttggcgt	aatcatgggtc	atagctgttt	cctgtgtgaa	attgttatcc	760
gctcacaatt	ccacacaaca	tacgagccgg	aagcataaag	tgtaaagcct	ggggtgccta	820
atgagtgagc	taactcacat	taattgcgtt	gcgctcactg	cccgttttcc	agtcgggaaa	880
cctgtcgtgc	cagctgcatt	aatgaatcgg	ccaacgcgcg	gggagaggcg	gtttgcgtat	940
tgggcgctct	tccgcttcct	cgctcactga	ctcgtgcgc	tcggtcgttc	ggctgcggcg	000
agcggtatca	gctcactcaa	aggcggtaat	acggttatcc	acagaatcag	gggataacgc	060
aggaaagaac	atgtgagcaa	aaggccagca	aaaggccagg	aaccgtaaaa	aggccgcgtt	120
gctggcggtt	ttccataggg	tccgcccccc	tgacgagcat	cacaaaaatc	gacgctcaag	180

tcagaggtgg	cgaaacccga	caggactata	aagataccag	gcgtttcccc	ctggaagctc	240
cctcgtgogc	tctcctgttc	cgaccctgcc	gcttaccgga	tacctgtccg	ccttttctccc	300
ttcgggaagc	gtggcgcttt	ctcaatgctc	acgctgtagg	tatctcagtt	cggtgtaggt	360
cgttcgctcc	aagctgggct	gtgtgcacga	acccccggt	cagcccgacc	gctgcgcctt	420
atccggtaac	tatcgtcttg	agtccaaccc	ggtaagacac	gacttatcgc	cactggcagc	480
agccactggc	aacaggatta	gcagagcgag	gtatgtaggc	gggtgctacag	agttcttgaa	540
gtgggtggcct	aactacggct	acactagaag	gacagtattt	ggtatctgcg	ctctgctgaa	600
gccagttacc	ttcggaaaaa	gagttggtag	ctcttgatcc	ggcaaacaaa	ccaccgctgg	660
tagcggtggt	ttttttgttt	gcaagcagca	gattacgcgc	agaaaaaaag	gatctcaaga	720
agatcctttg	atcttttcta	cggggtctga	cgctcagtg	aacgaaaact	cacgtaaggt	780
gatttttggtc	atgagattat	caaaaaggat	cttcacctag	atccttttaa	attaaaaatg	840
aagttttaaa	tcaatctaaa	gtatatatga	gtaaacttgg	tctgacagtt	accaatgctt	900
aatcagttag	gcacctatct	cagcgatctg	tctatttcgt	tcatccatag	ttgcctgact	960
ccccgtcgtg	tagataacta	cgatacggga	gggcttacca	tctggcccca	gtgctgcaat	020
gataccgcga	gaccacgct	caccggctcc	agatttatca	gcaataaacc	agccagccgg	080
aagggccgag	cgcagaagtg	gtcctgcaac	tttatccgcc	tccatccagt	ctattaattg	140
ttgccgggaa	gctagagtaa	gtagtctgcc	agttaatagt	ttgcgcaacg	ttgttgccat	200
tgctacaggc	atcgtgggtg	cacgctcgct	gtttggtatg	gcttcattca	gctccggttc	260
ccaacgatca	aggcgagtta	catgatcccc	catggttggtc	aaaaaagcgg	ttagctcctt	320
cggtcctccg	atcgttgtca	gaagtaagtt	ggccgcagtg	ttatcactca	tggttatggc	380
agcactgcat	aattctctta	ctgtcatgcc	atccgtaaga	tgcttttctg	tgactgggtga	440
gtactcaacc	aagtcattct	gagaatagt	tatgcggcga	ccgagttgct	cttgcccggc	500
gtcaatacgg	gataataccg	cgccacatag	cagaacttta	aaagtgtca	tcattggaaa	560
acgttcttcg	gggcgaaaac	tctcaaggat	cttaccgctg	ttgagatcca	gttcgatgta	620
accactcgt	gcacccaact	gatcttcagc	atcttttact	ttcaccagcg	tttctgggtg	680
agcaaaaaca	ggaaggcaaa	atgccgcaaa	aaaggggaata	agggcgacac	ggaaatgttg	740
aatactcata	ctcttccttt	ttcaatatta	ttgaagcatt	tatcagggtt	attgtctcat	800
gagcggatac	atatttgaat	gtatttagaa	aaataaacia	ataggggttc	cgcgcacatt	860
tccccgaaaa	gtgccacctg	acgtctaaga	aaccattatt	atcatgacat	taacctataa	920
aaataggcgt	atcacgaggc	cctttcgtc				949

<210> 23

<211> 18

<212> DNA

<213> PArtificial Sequence

<220>

<223> PCR primer

<400> 23

accctggcgc ccaatacg

18

<210> 24

<211> 18

<212> DNA

<213> Artificial Sequence

<220>

<223> SLF-A PCR primer

<400> 24

ctaactctag ctgcattg

18

<210> 25
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> ScBos1-1 oligo

<400> 25
gcggctcgag gggttttctc tcaacattg 29

<210> 26
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> ScBos1-2 oligo

<400> 26
gatcgcggcc gcgtaaggct tattgctgcg 30

<210> 27
<211> 35
<212> DNA
<213> Artificial Sequence

<220>
<223> ScBosldeIr oligo

<400> 27
ccaccaacgt tcctcacagc atggttgtaa agagc 35

<210> 28
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> ScBosldelf oligo

<400> 28
aggaacgttg gtggtgcg 18

<210> 29
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> oligo tRNAw

<400> 29
ggaatttcca agatttaatt ggagtcgaaa gctcgccotta

40

<210> 30
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer D

<220>
<221> modified_base
<222> (1)...(1)
<223> n is 3' end of HIS3-n

<220>
<221> unsure
<222> (1)...(20)
<223> n is a or g or c or t/u

<400> 30
nnnnnnnnnn nnnnnnnnnn

20

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/16271

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12N 15/74; C12Q 1/68

US CL : 435/6, 483

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 483

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
None

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6,004,779A (BRADLEY et al.) 21 December 1999, entire document, especially columns 9-20.	1-43

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T" Later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"A" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

24 JULY 2001

Date of mailing of the international search report

29 AUG 2001

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

IREM YUCEL

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/16271

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

US Patents, Derwent, JPO, EPO, STN (CAPLUS, Medline)

terms: yeast, cerevisiae, transcriptional, repressor, protein, polypeptide, metal ion, responsive, induc?, control?, regulat?, biomineralization, ANB1, ROX, SLF1, CYC8, LEXA, UBR1

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☒ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.